(12) UK Patent Application (19) GB (11) 2 296 243 (13) A

(43) Date of Printing by UK Office 26.06.1996

(21) Application No 9605354.1

(22) Date of Filing 15.10.1994

(30) Priority Data
(31) 9321302 (32) 15.10.1993 (33) GB

(86) International Application Data
PCT/EP94/03398 En 15.10.1994

(87) International Publication Data
WO95/10617 En 20.04.1995

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(51) INT CL^6
C12N 9/88 9/00 15/60 // (C12N 15/60 C12R 1:685 1:84) (C12N 9/88 C12R 1:645)

(52) UK CL (Edition O)
C3H HB7E HC1 H655 H740 H770
C6Y YF Y305 Y327 Y340 Y501 Y503
U1S S1068 S1275 S1284 S1289 S1333 S1334 S1517

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Biochim.Biophys.Acta 1993,1156(3),313-320 Planta 1993,191(1),137-142 Phytochemistry 1988,27 (11), 3401-3404

(58) Field of Search by ISA
INT CL^6 C12N , C12P

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(54) **Alpha-1,4-glucan lyase from a fungus,its purification gene cloning and expression in microorganisms**

(57) A method of preparing α-1,4-glucan lyase enzymes is described. The method comprises isolating the enzymes from a culture of a fungus wherein the culture is substantially free of any other organism. Also described are the amino acid sequences for the enzymes and their coding sequences.

GB 2 296 243 A

PCT

2296243

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/60, 9/00, 9/88

A3

(11) International Publication Number: WO 95/10617

(43) International Publication Date: 20 April 1995 (20.04.95)

(21) International Application Number: PCT/EP94/03398

(22) International Filing Date: 15 October 1994 (15.10.94)

(30) Priority Data:
9321302.3 15 October 1993 (15.10.93) GB

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 22 June 1995 (22.06.95)

(54) Title: ALPHA-1,4-GLUCAN LYASE FROM A FUNGUS, ITS PURIFICATION GENE CLONING AND EXPRESSION IN MICROORGANISMS

(57) Abstract

A method of preparing α-1,4-glucan lyase enzymes is described. The method comprises isolating the enzymes from a culture of a fungus wherein the culture is substantially free of any other organism. Also described are the amino acid sequences for the enzymes and their coding sequences.

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ALPHA-1,4-GLUCAN LYASE FROM A FUNGUS, ITS PURIFICATION GENE CLONING AND EXPRESSION IN MICROORGANISMS

The present invention relates to an enzyme, in particular α-1,4-glucan lyase ("GL"). The present invention also relates to a method of extracting same.

FR-A-2617502 and Baute et al in Phytochemistry [1988] vol. 27 No.11 pp3401-3403 report on the production of 1,5-D-anhydrofructose ("AF") in *Morchella vulgaris* by an apparent enzymatic reaction. The yield of production of AF is quite low. Despite a reference to a possible enzymatic reaction, neither of these two documents presents any amino acid sequence data for any enzyme let alone any nucleotide sequence information. These documents say that AF can be a precursor for the preparation of the antibiotic pyrone microthecin.

Yu et al in Biochimica et Biophysica Acta [1993] vol 1156 pp313-320 report on the preparation of GL from red seaweed and its use to degrade α-1,4-glucan to produce AF. The yield of production of AF is quite low. Despite a reference to the enzyme GL this document does not present any amino acid sequence data for that enzyme let alone any nucleotide sequence information coding for the same. This document also suggests that the source of GL is just algal.

According to the present invention there is provided a method of preparing the enzyme α-1,4-glucan lyase comprising isolating the enzyme from a culture of a fungus wherein the culture is substantially free of any other organism.

Preferably the enzyme is isolated and/or further purified using a gel that is not degraded by the enzyme.

Preferably the gel is based on dextrin or derivatives thereof, preferably a cyclodextrin, more preferably beta-cyclodextrin.

According to the present invention there is also provided a GL enzyme prepared by the method of the present invention.

Preferably the fungus is *Morchella costata* or *Morchella vulgaris*.

Preferably the enzyme comprises the amino acid sequence SEQ. ID. No. 1 or SEQ. I.D. No. 2, or any variant thereof.

The term "any variant thereof" means any substitution of, variation of, modification of, replacement of, deletion of or addition of an amino acid from or to the sequence providing the resultant enzyme has lyase activity.

According to the present invention there is also provided a nucleotide sequence coding for the enzyme α-1,4-glucan lyase, preferably wherein the sequence is not in its natural enviroment (i.e. it does not form part of the natural genome of a cellular organism expressing the enzyme).

Preferably the nucleotide sequence is a DNA sequence.

Preferably the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitution(s) for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

The expression "substantial homology" covers homology with respect to structure and/or nucleotide components and/or biological activity.

The expression "contains any suitable codon substitutions" covers any codon replacement or substitution with another codon coding for the same amino acid or any addition or removal thereof providing the resultant enzyme has lyase activity.

In other words, the present invention also covers a modified DNA sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode a polypeptide having the activity of a glucan lyase, preferably an enzyme having an increased lyase activity.

According to the present invention there is also provided a method of preparing the enzyme α-1,4-glucan lyase comprising expressing the nucleotide sequence of the present invention.

According to the present invention there is also provided the use of beta-cyclodextrin to purify an enzyme, preferably GL.

According to the present invention there is also provided a nucleotide sequence wherein the DNA sequence is made up of at least a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4, preferably wherein the sequence is in isolated form.

The present invention therefore relates to the isolation of the enzyme α-1,4-glucan lyase from a fungus. For example, the fungus can be any one of *Discina perlata, Discina parma, Gyromitra gigas, Gyromitra infula, Mitrophora hybrida, Morchella conica, Morchella costata, Morchella elata, Morchella hortensis, Morchella rotunda, Morchella vulgaris, Peziza badia, Sarcosphaera eximia, Disciotis venosa, Gyromitra esculenta, Helvella crispa, Helvella lacunosa, Leptopodia elastica, Verpa digitaliformis*, and other forms of *Morchella*. Preferably the fungus is *Morchella costata* or *Morchella vulgaris*.

The initial enzyme purification can be performed by the method as described by Yu et al (ibid).

However, preferably, the initial enzyme purification includes an optimized procedure in which a solid support is used that does not decompose under the purification step. This gel support further has the advantage that it is compatible with standard laboratory protein purification equipment.

The details of this optimized purification strategy are given later on. The purification is terminated by known standard techniques for protein purification.

The purity of the enzyme can be readily established using complementary electrophoretic techniques.

The purified lyase GL has been characterized according to pI, temperature- and pH-optima.

In this regard the fungal lyase shows a pI around 5.4 as determined by isoelectric focusing on gels with pH gradient of 3 to 9. The molecular weight determined by SDS-PAGE on 8-25% gradient gels was 110 kDa. The enzyme exhibits a pH optimum in the range pH 5-7. The temperature optimum was found to lay between 30-45°C.

GL sources	Optimal pH	Optimal pH range	Optimal temperature
M. costata	6.5	5.5-7.5	37 C; 40 C[a]
M. vulgaris	6.4	5.9-7.6	43 C; 48 C[a]

Parameters determined using glycogen as substrate; other parameters determined using amylopectin as substrate.

In a preferred embodiment the α-1,4-glucan lyase is purified from the fungus *Morchella costata* by affinity chromatography on β-cyclodextrin Sepharose, ion exchange on Mono Q HR 5/5 and gel filtration on Superose 12 columns.

PAS staining indicates that the fungal lyase was not glycosylated. In the cell-free fungus extract, only one form of α-1,4-glucan lyase was detected by activity gel staining on electrophoresis gels.

The enzyme should preferably be secreted to ease its purification. To do so the DNA encoding the mature enzyme is fused to a signal sequence, a promoter and a terminator from the chosen host.

For expression in *Aspergillus niger* the gpdA (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase - such as SEQ I.D. No. 3 or SEQ. I.D. No.4. The terminator sequence from the *A. niger* trpC gene is placed 3' to the gene (Punt, P.J. et al (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker for *A. niger*. Examples of selection markers for *A. niger* are the amdS gene, the argB gene, the pyrG gene, the hygB gene, the BmlR gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants.

The construction can be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the culture medium (Archer D.B. et al (1992): Biotechnol. Lett. 14, 357-362).

The amino acid composition can be established according to the method of Barholt and Jensen (Anal Biochem [1989] vol 177 pp 318-322). The sample for the amino acid analysis of the purified enzyme can contain 69ug/ml protein.

The amino acid sequence of the GL enzymes according to the present invention are shown in SEQ. I.D. No.1 and SEQ. I.D. No.2.

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 3 October 1994:

E. Coli containing plasmid pMC (NCIMB 40687) - [ref. DH5alpha-pMC];

E. Coli containing plasmid pMV1 (NCIMB 40688) - [ref. DH5alpha-pMV1]; and

E. Coli containing plasmid pMV2 (NCIMB 40689) - [ref. DH5alpha-pMV2].

Plasmid pMC is a pBluescript II KS containing a 4.1 kb fragment isolated from a genomic library constructed from *Morchella costata*. The fragment contains a gene coding for α-1,4-glucan lyase.

Plasmid pMV1 is a pBluescript II KS containing a 2.45 kb fragment isolated from a genomic library constructed from *Morchella vulgaris*. The fragment contains the 5' end of a gene coding for α-1,4-glucan lyase.

Plasmid MV2 is a pPUC19 containing a 3.1 kb fragment isolated from a genomic library constructed from *Morchella vulgaris*. The fragment contains the 3' end of a gene coding for α-1,4-glucan lyase.

In the following discussion, MC represents *Morchella costata* and MV represents *Morchella vulgaris*.

As mentioned, the GL coding sequence from *Morchella vulgaris* was contained in two plasmids. With reference to Figure 5 (discussed later) pMV1 contains the nucleotides from position 454 to position 2902; and pMV2 contains the nucleotides downstream from (and including) position 2897. With reference to Figures 2 and 3 (discussed later), to ligate the coding sequences one can digest pMV2 with restriction enzymes EcoRI and BamHI and then insert the relevant fragment into pMV1 digested with restriction enzymes EcoRI and BamHI.

Thus highly preferred embodiments of the present invention include a GL enzyme obtainable from the expression of the GL coding sequences present in plasmids that are the subject of either deposit NCIMB 40687 or deposit NCIMB 40688 and deposit

NCIMB 40689.

The present invention will now be described only by way of example.

In the following Examples reference is made to the accompanying figures in which:

Figure 1 shows a plasmid map of pMC;

Figure 2 shows a plasmid map of pMV1;

Figure 3 shows a plasmid map of pMV2;

Figure 4 shows the GL coding sequence and part of the 5' and 3' non-translated regions for genomic DNA obtained from *Morchella costata*;

Figure 5 shows the GL coding sequence and part of the 5' and 3' non-translated regions for genomic DNA obtained from *Morchella vulgaris*;

Figure 6 shows a comparison of the GL coding sequences and non-translated regions from *Morchella costata* and *Morchella vulgaris*;

Figure 7 shows the amino acid sequence represented as SEQ. I.D. No. 1 showing positions of the peptide fragments that were sequenced; and

Figure 8 shows the amino acid sequence represented as SEQ. I.D. No. 2 showing positions of the peptide fragments that were sequenced.

In more detail, in Figure 4, the total number of bases is 4726 - and the DNA sequence composition is: 1336 A; 1070 C; 1051 G; 1269 T. The ATG start codon is shown in bold. The introns are underlined. The stop codon is shown in italics.

In Figure 5, the total number of bases is 4670 - and the DNA sequence composition is: 1253 A; 1072 C; 1080 G; 1265 T. The ATG start codon is shown in bold. The introns are underlined. The stop codon is shown in italics.

In Figure 6, the two aligned sequences are those obtained from MC (total number of residues: 1066) and MV (total number of residues: 1070). The comparison matrix used was a structure-genetic matrix (Open gap cost: 10; Unit gap cost : 2). In this Figure, the character to show that two aligned residues are identical is ':'. The character to show that two aligned residues are similar is '.'. The amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. Overall there is: Identity: 920 (86.30%); Similarity: 51 (4.78%). The number of gaps inserted in MC is 1 and the number of gaps inserted in MV is 1.

In the attached sequence listings: SEQ. I.D.No. 1 is the amino-acid sequence for GL obtained from *Morchella costata*; SEQ. I.D.No. 2 is the amino-acid sequence for GL obtained from *Morchella vulgaris*; SEQ. I.D.No. 3 is the nucleotide coding sequence for GL obtained from *Morchella costata;* and SEQ. I.D.No. 4 is the nucleotide coding sequence for GL obtained from *Morchella vulgaris*.

In SEQ. I.D. No. 1 the total number of residues is 1066. The GL enzyme has an amino acid composition of:

46 Ala	13 Cys	25 His	18 Met	73 Thr
50 Arg	37 Gln	54 Ile	43 Phe	23 Trp
56 Asn	55 Glu	70 Leu	56 Pro	71 Tyr
75 Asp	89 Gly	71 Lys	63 Ser	78 Val

In SEQ.I.D. No. 2 the total number of residues is 1070. The GL enzyme has an amino acid composition of:

51 Ala	13 Cys	22 His	17 Met	71 Thr
50 Arg	40 Gln	57 Ile	45 Phe	24 Trp
62 Asn	58 Glu	74 Leu	62 Pro	69 Tyr
74 Asp	87 Gly	61 Lys	55 Ser	78 Val

1. ENZYME PURIFICATION AND CHARACTERIZATION OF THE α-1,4-GLUCAN LYASE FROM THE FUNGUS *MORCHELLA COSTATA*

1.1 Materials and Methods

The fungus *Morchella costata* was obtained from American Type Culture Collection (ATCC). The fungus was grown at 25°C on a shaker using the culture medium recommended by ATCC. The mycelia were harvested by filtration and washed with 0.9% NaCl.

The fungal cells were broken by homogenization followed by sonication on ice for 6x3 min in 50 mM citrate-NaOH pH 6.2 (Buffer A). Cell debris were removed by centrifugation at 25,000xg for 40 min. The supernatant obtained at this procedure was regarded as cell-free extract and was used for activity staining and Western blotting after separation on 8-25% gradient gels.

1.2 Separation by β-cyclodextrin Sepharose gel

The cell-free extract was applied directly to a β-cyclodextrin Sepharose gel 4B clolumn (2.6 x 18 cm) pre equilibrated with Buffer A. The column was washed with 3 volumes of Buffer A and 2 volumes of Buffer A containing 1 M NaCl. α-1,4-glucan lyase was eluted with 2 % dextrins in Buffer A. Active fractions were pooled and the buffer changed to 20 mM Bis-tris propane-HCl (pH 7.0, Buffer B).

Active fractions were applied onto a Mono Q HR 5/5 column pre-equilibrated with Buffer B. The fungal lyase was eluted with Buffer B in a linear gradient of 0.3 M NaCl.

The lyase preparation obtained after β-cyclodextrin Sepharose chromatography was alternatively concentrated to 150 µl and applied on a Superose 12 column operated under FPLC conditions.

1.3 Assay for α-1,4-glucan lyase activity and conditions for determination of substrate specificity, pH and temperature optimum

The reaction mixture for the assay of the α-1,4-glucan lyase activity contained 10 mg ml^{-1} amylopectin and 25 mM Mes-NaOH (pH 6.0).

The reaction was carried out at 30 °C for 30 min and stopped by the addition of 3,5-dinitrosalicylic acid reagent. Optical density at 550nm was measured after standing at room temperature for 10 min. 10 mM EDTA was added to the assay mixture when cell-free extracts were used.

The substrate amylopectin in the assay mixture may be replaced with other substrates and the reaction temperature may vary as specified in the text.

In the pH optimum investigations, the reaction mixture contained amylopection or maltotetraose 10 mg ml^{-1} in a 40 mM buffer. The buffers used were glycine-NaOH (pH 2.0-3.5), HoAc-NaoAc (pH 3.5-5.5), Mes-NaOH (pH 5.5-6.7), Mops-NaOH (6.0-8.0) and bicine-NaOH (7.6-9.0). The reactions were carried out at 30 °C for 30 min. The reaction conditions in the temperature optimum investigations was the same as above except that the buffer Mops-NaOH (pH 6.0) was used in all experiments. The reaction temperature was varied as indicated in the text.

SDS-PAGE, Native-PAGE and isoelectrofocusing were performed on PhastSystem (Pharmacia, Sweden) using 8-25% gradient gels and gels with a pH gradient of 3-9, respectively. Following electrophoresis, the gels were stained by silver staining according to the procedures recommended by the manufacturer (Pharmacia). The glycoproteins were stained by PAS adapted to the PhastSystem. For activity staining, the electrophoresis was performed under native conditions at 6°C.

Following the electrophoresis, the gel was incubated in the presence of 1% soluble starch at 30°C overnight. Activity band of the fungal lyase was revealed by staining with I_2/KI solution.

1.4 Results

1.4.1 Purification, molecular mass and isoelectric point of the α-1,4-glucan lyase

The fungal lyase was found to adsorb on columns packed with β-cyclodextrin Sepharose, starches and Red Sepharose. Columns packed with β-cyclodextrin Sepharose 4B gel and starches were used for purification purposes.

The lyase preparation obtained by this step contained only minor contaminating proteins having a molecular mass higher than the fungal lyase. The impurity was either removed by ion exchange chromatography on Mono Q HR 5/5 or more efficiently by gel filtration on Superose 12.

The purified enzyme appeared colourless and showed no absorbance in the visible light region. The molecular mass was determined to 110 kDa as estimated on SDS-PAGE.

The purified fungal lyase showed a isoelectric point of pI 5.4 determined by isoelectric focusing on gels with a pH gradient of 3 to 9. In the native electrophoresis gels, the enzyme appeared as one single band. This band showed starch-degrading activity as detected by activity staining. Depending the age of the

culture from which the enzyme is extracted, the enzyme on the native and isoelectric focusing gels showed either as a sharp band or a more diffused band with the same migration rate and pI.

1.4.2 The pH and temperature optimum of the fungal lyase catalayzed reaction

The pH optimum pH range for the fungal lyase catalyzed reaction was found to be between pH 5 and pH 7.

1.4.3 Substrate specificity

The purified fungal lyase degraded maltosaccharides from maltose to maltoheptaose. However, the degradation rates varied. The highest activity achieved was with maltotetraose (activity as 100%), followed by maltohexaose (97%), maltoheptaose (76%), maltotriose (56%) and the lowest activity was observed with maltose (2%).

Amylopectin, amylose and glycogen were also degraded by the fungal lyase (% will be determined). The fungal lyase was an exo-lyase, not a endolyase as it degraded p-nitrophenyl α-D-maltoheptaose but failed to degrade reducing end blocked p-nitrophenyl α-D-maltoheptaose.

1.5 *Morchella Vulgaris*

The protocols for the enzyme purification and charaterisation of alpha 1,4-glucal lyase obtained from *Morchella Vulgaris* were the same as those above for *Morchella Costata* (with similar results - see results mentioned above).

2. AMINO ACID SEQUENCING OF THE α-1,4-GLUCAN LYASE FROM FUNGUS

2.1 Amino acid sequencing of the lyases

The lyases were digested with either endoproteinase Arg-C from *Clostridium histolyticum* or endoproteinase Lys-C from *Lysobacter enzymogenes*, both sequencing grade purchased from Boehringer Mannheim, Germany. For digestion with endoproteinase Arg-C, freezedried lyase (0.1 mg) was dissolved in 50 µl 10 M urea, 50 mM methylamine, 0.1 M Tris-HCl, pH 7.6. After overlay with N_2 and addition of 10 µl of 50 mM DTT and 5 mM EDTA the protein was denatured and reduced for 10 min at 50°C under N_2. Subsequently, 1 µg of endoproteinase Arg-C in 10 µl of 50 mM Tris-HCl, pH 8.0 was added, N_2 was overlayed and the digestion was carried out for 6h at 37°C.

For subsequent cysteine derivatization, 12.5 µl 100 mM iodoacetamide was added and the solution was incubated for 15 min at RT in the dark under N_2.

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved in 50 µl of 8 M urea, 0.4 M NH_4HCO_3, pH 8.4. After overlay with N_2 and addition of 5 µl of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N_2. After cooling to RT, 5 µl of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at RT in the dark under N_2. Subsequently, 90 µl of water and 5 µg of endoproteinase Lys-C in 50 µl of 50 mM tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N_2.

The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 µm; The Separations Group; California) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 µm; Dr. Ole Schou, Novo Nordisk, Denmark) using the same solvent system prior to sequencing on an

Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

The amino acid sequence information from the enzyme derived from the fungus *Morchella costata* is shown Fig. 7.

The amino acid sequence information from the enzyme derived from the fungus *Morchella vulgaris* is shown Fig. 8.

3. DNA SEQUENCING OF GENES CODING FOR THE α-1,4-GLUCAN LYASE FROM FUNGUS

3.1 METHODS FOR MOLECULAR BIOLOGY

DNA was isolated as described by Dellaporte et al (1983 - Plant Mol Biol Rep vol 1 pp19-21).

3.2 PCR

The preparation of the relevant DNA molecule was done by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufactures instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

PCR cycles:

no of cycles	C	time (min.)
1	98	5
	60	5
addition of Taq polymerase and oil		
35	94	1
	47	2
	72	3
1	72	20

3.3 CLONING OF PCR FRAGMENTS

PCR fragments were cloned into pT7Blue (from Novagen) following the instructions of the supplier.

3.4 DNA SEQUENCING

Double stranded DNA was sequenced essentially according to the dideoxy method of Sanger et al. (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson, A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.)

3.5 SCREENING OF THE LIBRARIES

Screening of the Lambda Zap libraries obtained from Stratagene, was performed in accordance with the manufacturer's instructions except that the prehybridization and hybridization was performed in 2xSSC, 0.1% SDS, 10xDenhardt's and 100μg/ml denatured salmon sperm DNA.

To the hybridization solution a 32P-labeled denatured probe was added. Hybridization was performed over night at 55°C. The filters were washed twice in 2xSSC, 0.1% SDS and twice in 1xSSC, 0.1% SDS.

3.6 PROBE

The cloned PCR fragments were isolated from the pT7blue vector by digestion with appropriate restriction enzymes. The fragments were seperated from the vector by agarose gel electrophoresis and the fragments were purified from the agarose by Agarase (Boehringer Mannheim). As the fragments were only 90-240 bp long the isolated fragments were exposed to a ligation reaction before labelling with 32P-dCTP using either Prime-It random primer kit (Stratagene) or Ready to Go DNA labelling

kit (Pharmacia).

3.7 RESULTS

3.7.1 Generation of PCR DNA fragments coding for α-1,4-glucan lyase.

The amino acid sequences (shown below) of three overlapping tryptic peptides from α-1,4-glucan lyase were used to generate mixed oligonucleotides, which could be used as PCR primers for amplification of DNA isolated from both MC and MV.

Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr Val Leu Asp Ile Val Lys
Pro Gly His Gly Glu Tyr Val Gly Trp Gly Glu Met Gly Gly Ile Gln Phe Met Lys
Glu Pro Thr Phe Met Asn Tyr Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr
Ala Gln Gly Ala Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr

In the first PCR amplification primers A1/A2 (see below) were used as upstream primers and primers B1/B2 (see below) were used as downstream primer.

Primer A1: CA(GA)CA(CT)AA(GA)ATGCT(GATC)AA(GA)GA(CT)AC
Primer A2: CA(GA)CA(CT)AA(GA)ATGTT(GA)AA(GA)GA(CT)AC
Primer B1: TA(GA)AA(GATC)GG(GA)TC(GA)CT(GA)TG(GA)TA
Primer B2: TA(GA)AA(GATC)GG(GA)TC(GATC)GA(GA)TG(GA)TA

The PCR products were analysed on a 2% LMT agarose gel and fragments of the expected sizes were cut out from the gel and treated with Agarase (Boehringer Manheim) and cloned into the pT7blue Vector (Novagen) and sequenced.

The cloned fragments from the PCR amplification coded for amino acids corresponding to the sequenced peptides (see above) and in each case in addition to two intron sequences. For MC the PCR amplified DNA sequence corresponds to the sequence shown as from position 1202 to position 1522 with reference to Figure 4. For MV the PCR amplified DNA sequence corresponds to the sequence shown as

from position 1218 to position 1535 with reference to Figure 5.

3.7.2 Screening of the genomic libraries with the cloned PCR fragments.

Screening of the libraries with the above-mentioned clone gave two clones for each source. For MC the two clones were combined to form the sequence shown in Figure 4 (see below). For MV the two clones could be combined to form the sequence shown in Figure 5 in the manner described above.

An additional PCR was performed to supplement the MC clone with PstI, PvuII, AscI and NcoI restriction sites immediately in front of the ATG start codon using the following oligonucleotide as an upstream primer:
AAACTGCAGCTGGCGCGCCATGGCAGGATTTTCTGAT
and a primer containing the complement sequence of bp 1297-1318 in Figure 4 was used as a downstream primer.

The complete sequence for MC was generated by cloning the 5' end of the gene as a BglII-EcoRI fragment from one of the genomic clone (first clone) into the BamHI-EcoRI sites of pBluescript II KS+ vector from Stratagene. The 3' end of the gene was then cloned into the modified pBluescript II KS+ vector by ligating an NspV (blunt ended, using the DNA blunting kit from Amersham International)-EcoRI fragment from the other genomic clone (second clone) after the modified pBluescript II KS+ vector had been digested with EcoRI and EcoRV. Then the intermediate part of the gene was cloned in to the further modified pBluescript II KS+ vector as an EcoRI fragment from the first clone by ligating that fragment into the further modified pBluescript II KS+ vector digested with EcoRI.

4. EXPRESSION OF THE GL GENE IN MICRO-ORGANISMS

The DNA sequence encoding the GL can be introduced into microorganisms to produce the enzyme with high specific activity and in large quantities.

In this regard, the MC gene (Figure 4) was cloned as a XbaI-XhoI blunt ended (using the DNA blunting kit from Amersham International) fragment into the *Pichia* expression vector pHIL-D2 (containing the AOX1 promoter) digested with EcoRI and blunt ended (using the DNA blunting kit from Amersham International) for expression in *Pichia pastoris* (according to the protocol stated in the *Pichia* Expression Kit supplied by Invitrogen).

In another embodiment, the MC gene 1 (same as Figure 4 except that it was modified by PCR to introduce restriction sites as described above) was cloned as a PvuII-XhoI blunt ended fragment (using the DNA blunting kit from Amersham International) into the Aspergillus expression vector pBARMTE1 (containing the methyl tryptophan resistance promoter from *Neuropera crassa*) digested with SmaI for expression in *Aspergillus niger* (Pall et al (1993) Fungal Genet Newslett. vol 40 pages 59-62). The protoplasts were prepared according to Daboussi et al (Curr Genet (1989) vol 15 pp 453-456) using lysing enzymes Sigma L-2773 and the lyticase Sigma L-8012. The transformation of the protoplasts was followed according to the protocol stated by Buxton et al (Gene (1985) vol 37 pp 207-214) except that for plating the transformed protoplasts the protocol laid out in Punt et al (Methods in Enzymology (1992) vol 216 pp 447 - 457) was followed but with the use of 0.6% osmotic stabilised top agarose.

The results showed that lyase activity was observed in the transformed *Pichia pastoris* and *Aspergillus niger*. These experiments are now described.

ANALYSES OF PICHIA LYASE TRANSFORMANTS AND *ASPERGILLUS* LYASE TRANSFORMANTS

GENERAL METHODS

Preparation of cell-free extracts.

The cells were harvested by centrifugation at 9000 rpm for 5 min and washed with 0.9% NaCl and resuspended in the breaking buffer (50mM K-phosphate, pH 7.5

containing 1mM of EDTA, and 5% glycerol). Cells were broken using glass beads and vortex treatment. The breaking buffer contained 1 mM PMSF (protease inhibitor). The lyase extract (supernatant) was obtained after centrifugation at 9000 rpm for 5 min followed by centrifugation at 20,000 xg for 5min.

Assay of lyase activity by alkaline 3,5-dinitrosalicylic acid reagent (DNS)

One volume of lyase extract was mixed with an equal volume of 4% amylopectin solution. The reaction mixture was then incubated at a controlled temperature and samples vere removed at specified intervals and analyzed for AF.

The lyase activity was also analyzed using a radioactive method.

The reaction mixture contained 10 μl ^{14}C-starch solution (1 μCi; Sigma Chemicals Co.) and 10 μl of the lyase extract. The reaction mixture was left at 25°C overnight and was then analyzed in the usual TLC system. The radioactive AF produced was detected using an Instant Imager (Pachard Instrument Co., Inc., Meriden, CT).

Electrophoresis and Western blotting

SDS-PAGE was performed using 8-25% gradient gels and the PhastSystem (Pharmacia). Western blottings was also run on a Semidry transfer unit of the PhastSystem. Primary antibodies raised against the lyase purified from the red seaweed collected at Qingdao (China) were used in a dilution of 1:100. Pig antirabbit IgG conjugated to alkaline phosphatase (Dako A/S, Glostrup, Denmark) were used as secondary antibodies and used in a dilution of 1:1000.

Part I, Analysis of the Pichia transformantscontaining the above mentioned construct

MC-Lyase expressed intracellularly in *Pichia pastoris*

Names of culture	Specific activity*
A18	10
A20	32
A21	8
A22	8
A24	6

*The specific activity was defined as nmol of AF produced per min per mg protein at 25°C.

Part II, The *Aspergilus* transformants

Results

I. Lyase activity was determined after 5 days incubation(minimal medium containing 0.2% casein enzymatic hydrolysate analysis by the alkaline 3,5-dinitrosalicylic acid reagent

Lyase activity analysis in cell-free extracts

Name of the culture	Specific activity*
8.13	11
8.16	538
8.19	37

*The specific activity was defined as nmol of AF produced per min per mg protein at 25°C.

The results show that the MC-lyase was expressed intracellular in *A. niger*.

Instead of *Aspergillus niger* as host, other industrial important nicroorganisms for which good expression systems are known could be used such as: *Aspergillus oryzae, Aspergillus sp., Trichoderma sp., Saccharomyces cerevisiae, Kluyveromyces sp., Hansenula sp., Pichia sp., Bacillus subtilis, B. amyloliquefaciens, Bacillus sp., Streptomyces sp.* or *E. coli*.

Other preferred embodiments of the present invention include any one of the following: A transformed host organism having the capability of producing AF as a consequence of the introduction of a DNA sequence as herein described; such a transformed host organism which is a microorganism - preferably wherein the host organism is selected from the group consisting of bacteria, moulds, fungi and yeast;

preferably the host organism is selected from the group consisting of *Saccharomyces, Kluyveromyces, Aspergillus, Trichoderma Hansenula, Pichia, Bacillus Streptomyces, Eschericia* such as *Aspergillus oryzae, Saccharomyces cerevisiae, bacillus sublilis, Bacillus amyloliquefascien, Eschericia coli.*; A method for preparing the sugar 1,5-D-anhydrofructose comprising contacting an alpha 1,4-glucan (e.g. starch) with the enzyme α-1,4-glucan lyase expressed by a transformed host organism comprising a nucleotide sequence encoding the same, preferably wherein the nucleotide sequence is a DNA sequence, preferably wherein the DNA sequence is one of the sequences hereinbefore described; A vector incorporating a nucleotide sequence as hereinbefore described, preferably wherein the vector is a replication vector, preferably wherein the vector is an expression vector containing the nucleotide sequence downstream from a promoter sequence, preferably the vector contains a marker (such as a resistance marker); Cellular organisms, or cell line, transformed with such a vector; A method of producing the product α-1,4-glucan lyase or any nucleotide sequence or part thereof coding for same, which comprises culturing such an organism (or cells from a cell line) transfected with such a vector and recovering the product.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Danisco A/S
(B) STREET: Langebrogade 1
(C) CITY: Copenhagen
(D) STATE: Copenhagen K
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-1001
(ii) TITLE OF INVENTION: ENZYME
(iii) NUMBER OF SEQUENCES: 10
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/EP94/03398

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1066 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Ala Gly Phe Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
1 5 10 15

Tyr Ser Val Ala Leu Asp Trp Lys Gly Pro Gln Lys Ile Ile Gly Val
20 25 30

Asp Thr Thr Pro Pro Lys Ser Thr Lys Phe Pro Lys Asn Trp His Gly
35 40 45

Val Asn Leu Arg Phe Asp Asp Gly Thr Leu Gly Val Val Gln Phe Ile
50 55 60

Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Gly Phe Lys Thr Ser
65 70 75 80

Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
85 90 95

Ser Thr Leu Ser Asn Lys Leu Asp Thr Tyr Arg Gly Leu Thr Trp Glu
100 105 110

Thr Lys Cys Glu Asp Ser Gly Asp Phe Phe Thr Phe Ser Ser Lys Val
115 120 125

Thr Ala Val Glu Lys Ser Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
130 135 140

Leu Arg Ile His Leu Trp Lys Ser Pro Phe Arg Ile Gln Val Val Arg
145 150 155 160

Thr Leu Thr Pro Leu Lys Asp Pro Tyr Pro Ile Pro Asn Val Ala Ala
165 170 175

Ala Glu Ala Arg Val Ser Asp Lys Val Val Trp Gln Thr Ser Pro Lys
180 185 190

Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
195 200 205

Val Leu Asp Ile Val Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
210 215 220

Glu Met Gly Gly Ile Gln Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
225 230 235 240

Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
245 250 255

Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
260 265 270

Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
275 280 285

Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
290 295 300

Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
305 310 315 320

Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
325 330 335

Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
340 345 350

Tyr Gly Tyr Gln Gln Glu Ser Asp Leu Tyr Ser Val Val Gln Gln Tyr
355 360 365

Arg Asp Cys Lys Phe Pro Leu Asp Gly Ile His Val Asp Val Asp Val
370 375 380

Gln Asp Gly Phe Arg Thr Phe Thr Thr Asn Pro His Thr Phe Pro Asn
385 390 395 400

Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Asn Gly Ile Lys Cys Ser
405 410 415

Thr Asn Ile Thr Pro Val Ile Ser Ile Asn Asn Arg Glu Gly Gly Tyr
420 425 430

Ser Thr Leu Leu Glu Gly Val Asp Lys Lys Tyr Phe Ile Met Asp Asp
435 440 445

Arg Tyr Thr Glu Gly Thr Ser Gly Asn Ala Lys Asp Val Arg Tyr Met
450 455 460

Tyr Tyr Gly Gly Gly Asn Lys Val Glu Val Asp Pro Asn Asp Val Asn
465 470 475 480

Gly Arg Pro Asp Phe Lys Asp Asn Tyr Asp Phe Pro Ala Asn Phe Asn
485 490 495

Ser Lys Gln Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
500 505 510

Gly Ser Ala Gly Phe Tyr Pro Asp Leu Asn Arg Lys Glu Val Arg Ile
515 520 525

Trp Trp Gly Met Gln Tyr Lys Tyr Leu Phe Asp Met Gly Leu Glu Phe
530 535 540

Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Thr Ser Tyr Gly Asp
545 550 555 560

Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ser Asp Ser Val Thr
565 570 575

Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Thr Trp Ala Leu Tyr Ser
580 585 590

Tyr Asn Leu His Lys Ala Thr Trp His Gly Leu Ser Arg Leu Glu Ser
595 600 605

Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
610 615 620

Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Asn Trp
625 630 635 640

Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
645 650 655

Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Tyr Arg
660 665 670

Asp Ala Asn Gly Val Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile
675 680 685

Arg Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr
690 695 700

Val Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ser Tyr Pro Lys
705 710 715 720

His Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys
725 730 735

Ser Val Leu Glu Ile Cys Arg Tyr Tyr Val Glu Leu Arg Tyr Ser Leu
740 745 750

Ile Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met
755 760 765

Pro Ile Thr Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe
770 775 780

Phe Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp
785 790 795 800

Asp Ile Leu Val Ala Pro Ile Leu His Ser Arg Lys Glu Ile Pro Gly
805 810 815

Glu Asn Arg Asp Val Tyr Leu Pro Leu Tyr His Thr Trp Tyr Pro Ser
820 825 830

Asn Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val
835 840 845

Glu Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu
850 855 860

Asp Tyr Asn Leu Phe His Ser Val Val Pro Val Tyr Val Arg Glu Gly
865 870 875 880

Ala Ile Ile Pro Gln Ile Glu Val Arg Gln Trp Thr Gly Gln Gly Gly
885 890 895

Ala Asn Arg Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr
900 905 910

Cys Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Glu Asp
915 920 925

Leu Pro Gln Tyr Lys Glu Thr His Glu Gln Ser Lys Val Glu Gly Ala
930 935 940

Glu Ile Ala Lys Gln Ile Gly Lys Lys Thr Gly Tyr Asn Ile Ser Gly
945 950 955 960

Thr Asp Pro Glu Ala Lys Gly Tyr His Arg Lys Val Ala Val Thr Gln
965 970 975

Thr Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu Pro Lys His Asn
980 985 990

Gly Tyr Asp Pro Ser Lys Glu Val Gly Asp Tyr Tyr Thr Ile Ile Leu
995 1000 1005

Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp Val Ser Lys Thr
1010 1015 1020

Thr Val Asn Val Glu Gly Gly Val Glu His Gln Val Tyr Lys Asn Ser
1025 1030 1035 1040

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1045 1050 1055

Lys Ser Val Lys Ile Thr Cys Thr Ala Ala
1060 1065

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1070 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Gly Leu Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
1 5 10 15

Tyr Ala Ala Ala Lys Gly Trp Ser Gly Pro Gln Lys Ile Ile Arg Tyr
20 25 30

Asp Gln Thr Pro Pro Gln Gly Thr Lys Asp Pro Lys Ser Trp His Ala
35 40 45

Val Asn Leu Pro Phe Asp Asp Gly Thr Met Cys Val Val Gln Phe Val
50 55 60

Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Ser Val Lys Thr Ser
65 70 75 80

Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
85 90 95

Thr Thr Leu Val Gly Asn Leu Asp Ile Phe Arg Gly Leu Thr Trp Val
100 105 110

Ser Thr Leu Glu Asp Ser Gly Glu Tyr Tyr Thr Phe Lys Ser Glu Val
115 120 125

Thr Ala Val Asp Glu Thr Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
130 135 140

Leu Lys Ile Tyr Leu Trp Lys Asn Pro Phe Arg Ile Gln Val Val Arg
145 150 155 160

Leu Leu Thr Pro Leu Val Asp Pro Phe Pro Ile Pro Asn Val Ala Asn
165 170 175

Ala Thr Ala Arg Val Ala Asp Lys Val Val Trp Gln Thr Ser Pro Lys
180 185 190

Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
195 200 205

Val Leu Asp Ile Ile Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
210 215 220

Glu Met Gly Gly Ile Glu Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
225 230 235 240

Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
245 250 255

Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
260 265 270

Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
275 280 285

Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
290 295 300

Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
305 310 315 320

Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
325 330 335

Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
340 345 350

Tyr Gly Tyr Gln Gln Glu Ser Asp Leu His Ala Val Val Gln Gln Tyr
355 360 365

Arg Asp Thr Lys Phe Pro Leu Asp Gly Leu His Val Asp Val Asp Phe
370 375 380

Gln Asp Asn Phe Arg Thr Phe Thr Thr Asn Pro Ile Thr Phe Pro Asn
385 390 395 400

Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Asn Gly Ile Lys Cys Ser
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Thr Asn Ile Thr Pro Val Ile Ser Ile Arg Asp Arg Pro Asn Gly Tyr
420 425 430

Ser Thr Leu Asn Glu Gly Tyr Asp Lys Lys Tyr Phe Ile Met Asp Asp
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Arg Tyr Thr Glu Gly Thr Ser Gly Asp Pro Gln Asn Val Arg Tyr Ser
450 455 460

Phe Tyr Gly Gly Gly Asn Pro Val Glu Val Asn Pro Asn Asp Val Trp
465 470 475 480

Ala Arg Pro Asp Phe Gly Asp Asn Tyr Asp Phe Pro Thr Asn Phe Asn
485 490 495

Cys Lys Asp Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
500 505 510

Gly Thr Pro Gly Tyr Tyr Pro Asp Leu Asn Arg Glu Glu Val Arg Ile
515 520 525

Trp Trp Gly Leu Gln Tyr Glu Tyr Leu Phe Asn Met Gly Leu Glu Phe
530 535 540

Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Ser Ser Tyr Gly Asp
545 550 555 560

Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ala Asp Ser Val Thr
565 570 575

Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Ser Trp Ala Leu Tyr Ser
580 585 590

Tyr Asn Leu His Lys Ala Thr Phe His Gly Leu Gly Arg Leu Glu Ser
595 600 605

Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
610 615 620

Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Thr Trp
625 630 635 640

Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
645 650 655

Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Ala Arg
660 665 670

Thr Glu Ile Gly Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile Arg
675 680 685

Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr Val
690 695 700

Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ala Tyr Pro Lys His
705 710 715 720

Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys Ser
725 730 735

Val Leu Glu Ile Cys Arg Tyr Trp Val Glu Leu Arg Tyr Ser Leu Ile
740 745 750

Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met Pro
755 760 765

Leu Ala Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe Phe
770 775 780

Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp Asp
785 790 795 800

Ile Leu Val Ala Pro Ile Leu His Ser Arg Asn Glu Val Pro Gly Glu
805 810 815

Asn Arg Asp Val Tyr Leu Pro Leu Phe His Thr Trp Tyr Pro Ser Asn
820 825 830

Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val Glu
835 840 845

Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu Asp
850 855 860

Tyr Asn Leu Phe His Asn Val Val Pro Val Tyr Ile Arg Glu Gly Ala
865 870 875 880

Ile Ile Pro Gln Ile Gln Val Arg Gln Trp Ile Gly Glu Gly Gly Pro
885 890 895

Asn Pro Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr Val
900 905 910

Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Asp Asp Leu
915 920 925

Pro Gln Tyr Arg Glu Ala Tyr Glu Gln Ala Lys Val Glu Gly Lys Asp
930 935 940

Val Gln Lys Gln Leu Ala Val Ile Gln Gly Asn Lys Thr Asn Asp Phe
945 950 955 960

Ser Ala Ser Gly Ile Asp Lys Glu Ala Lys Gly Tyr His Arg Lys Val
965 970 975

Ser Ile Lys Gln Glu Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu
980 985 990

Pro Lys His Asn Gly Tyr Asp Pro Ser Lys Glu Val Gly Asn Tyr Tyr
995 1000 1005

Thr Ile Ile Leu Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp
1010 1015 1020

Val Ser Gln Ala Thr Val Asn Ile Glu Gly Gly Val Glu Cys Glu Ile
1025 1030 1035 1040

Phe Lys Asn Thr Gly Leu His Thr Val Val Val Asn Val Lys Glu Val
1045 1050 1055

Ile Gly Thr Thr Lys Ser Val Lys Ile Thr Cys Thr Thr Ala
1060 1065 1070

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3201 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGGCAGGAT TTTCTGATCC TCTCAACTTT TGCAAAGCAG AAGACTACTA CAGTGTTGCG 60

CTAGACTGGA AGGGCCCTCA AAAAATCATT GGAGTAGACA CTACTCCTCC AAAGAGCACC 120

AAGTTCCCCA AAAACTGGCA TGGAGTGAAC TTGAGATTCG ATGATGGGAC TTTAGGTGTG 180

GTTCAGTTCA TTAGGCCGTG CGTTTGGAGG GTTAGATACG ACCCTGGTTT CAAGACCTCT 240

GACGAGTATG GTGATGAGAA TACGAGGACA ATTGTGCAAG ATTATATGAG TACTCTGAGT 300

AATAAATTGG ATACTTATAG AGGTCTTACG TGGGAAACCA AGTGTGAGGA TTCGGGAGAT 360

TTCTTTACCT TCTCATCCAA GGTCACCGCC GTTGAAAAAT CCGAGCGGAC CCGCAACAAG 420

GTCGGCGATG GCCTCAGAAT TCACCTATGG AAAAGCCCTT TCCGCATCCA AGTAGTGCGC 480

ACCTTGACCC CTTTGAAGGA TCCTTACCCC ATTCCAAATG TAGCCGCAGC CGAAGCCCGT 540

GTGTCCGACA AGGTCGTTTG GCAAACGTCT CCCAAGACAT TCAGAAAGAA CCTGCATCCG 600

CAACACAAGA TGCTAAAGGA TACAGTTCTT GACATTGTCA AACCTGGACA TGGCGAGTAT 660

GTGGGGTGGG GAGAGATGGG AGGTATCCAG TTTATGAAGG AGCCAACATT CATGAACTAT 720

TTTAACTTCG ACAATATGCA ATACCAGCAA GTCTATGCCC AAGGTGCTCT CGATTCTCGC 780

GAGCCACTGT ACCACTCGGA TCCCTTCTAT CTTGATGTGA ACTCCAACCC GGAGCACAAG 840

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AAGCCCAGAT ATATTCTCGG GGCCCATCAA GCCTGTTATG GATACCAACA GGAAAGTGAC 1080

TTGTATTCTG TGGTCCAGCA GTACCGTGAC TGTAAATTTC CACTTGACGG GATTCACGTC 1140

GATGTCGATG TTCAGGACGG CTTCAGAACT TTCACCACCA ACCCACACAC TTTCCCTAAC 1200

CCCAAAGAGA TGTTTACTAA CTTGAGGAAT AATGGAATCA AGTGCTCCAC CAATATCACT 1260

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GTTCGGTACA TGTACTACGG TGGTGGTAAT AAGGTTGAGG TCGATCCTAA TGATGTTAAT 1440

GGTCGGCCAG ACTTTAAAGA CAACTATGAC TTCCCCGCGA ACTTCAACAG CAAACAATAC 1500

CCCTATCATG GTGGTGTGAG CTACGGTTAT GGGAACGGTA GTGCAGGTTT TTACCCGGAC 1560

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CATGGTCTTA GTCGTCTCGA ATCTCGTAAG AACAAACGAA ACTTCATCCT CGGGCGTGGA 1860

AGTTATGCCG GAGCCTATCG TTTTGCTGGT CTCTGGACTG GGGATAATGC AAGTAACTGG 1920

GAATTCTGGA AGATATCGGT CTCTCAAGTT CTTTCTCTGG GCCTCAATGG TGTGTGCATC 1980

GCGGGGTCTG ATACGGGTGG TTTTGAACCC TACCGTGATG CAAATGGGGT CGAGGAGAAA 2040

TACTGTAGCC CAGAGCTACT CATCAGGTGG TATACTGGTT CATTCCTCTT GCCGTGGCTC 2100

AGGAACCATT ATGTCAAAAA GGACAGGAAA TGGTTCCAGG AACCATACTC GTACCCCAAG 2160

CATCTTGAAA CCCATCCAGA ACTCGCAGAC CAAGCATGGC TCTATAAATC CGTTTTGGAG 2220

ATCTGTAGGT ACTATGTGGA GCTTAGATAC TCCCTCATCC AACTACTTTA CGACTGCATG 2280

TTTCAAAACG TAGTCGACGG TATGCCAATC ACCAGATCTA TGCTCTTGAC CGATACTGAG 2340

GATACCACCT TCTTCAACGA GAGCCAAAAG TTCCTCGACA ACCAATATAT GGCTGGTGAC 2400

GACATTCTTG TTGCACCCAT CCTCCACAGT CGCAAAGAAA TTCCAGGCGA AAACAGAGAT 2460

GTCTATCTCC CTCTTTACCA CACCTGGTAC CCCTCAAATT TGAGACCATG GGACGATCAA 2520

GGAGTCGCTT TGGGGAATCC TGTCGAAGGT GGTAGTGTCA TCAATTATAC TGCTAGGATT 2580

GTTGCACCCG AGGATTATAA TCTCTTCCAC AGCGTGGTAC CAGTCTACGT TAGAGAGGGT 2640

GCCATCATCC CGCAAATCGA AGTACGCCAA TGGACTGGCC AGGGGGGAGC CAACCGCATC 2700

AAGTTCAACA TCTACCCTGG AAAGGATAAG GAGTACTGTA CCTATCTTGA TGATGGTGTT 2760

AGCCGTGATA GTGCGCCGGA AGACCTCCCA CAGTACAAAG AGACCCACGA ACAGTCGAAG 2820

GTTGAAGGCG CGGAAATCGC AAAGCAGATT GGAAAGAAGA CGGGTTACAA CATCTCAGGA 2880

ACCGACCCAG AAGCAAAGGG TTATCACCGC AAAGTTGCTG TCACACAAAC GTCAAAAGAC 2940

AAGACGCGTA CTGTCACTAT TGAGCCAAAA CACAATGGAT ACGACCCTTC CAAAGAGGTG 3000

GGTGATTATT ATACCATCAT TCTTTGGTAC GCACCAGGTT TCGATGGCAG CATCGTCGAT 3060

GTGAGCAAGA CGACTGTGAA TGTTGAGGGT GGGGTGGAGC ACCAAGTTTA TAAGAACTCC 3120

GATTTACATA CGGTTGTTAT CGACGTGAAG GAGGTGATCG GTACCACAAA GAGCGTCAAG 3180

ATCACATGTA CTGCCGCTTA A 3201

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3213 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGGCAGGAT TATCCGACCC TCTCAATTTC TGCAAAGCAG AGGACTACTA CGCTGCTGCC 60

AAAGGCTGGA GTGGCCCTCA GAAGATCATT CGCTATGACC AGACCCCTCC TCAGGGTACA 120

AAAGATCCGA AAAGCTGGCA TGCGGTAAAC CTTCCTTTCG ATGACGGGAC TATGTGTGTA 180

GTGCAATTCG TCAGACCCTG TGTTTGGAGG GTTAGATATG ACCCCAGTGT CAAGACTTCT 240

GATGAGTACG GCGATGAGAA TACGAGGACT ATTGTACAAG ACTACATGAC TACTCTGGTT 300

GGAAACTTGG ACATTTTCAG AGGTCTTACG TGGGTTTCTA CGTTGGAGGA TTCGGGCGAG 360

TACTACACCT TCAAGTCCGA AGTCACTGCC GTGGACGAAA CCGAACGGAC TCGAAACAAG 420

GTCGGCGACG GCCTCAAGAT TTACCTATGG AAAAATCCCT TTCGCATCCA GGTAGTGCGT 480

CTCTTGACCC CCCTGGTGGA CCCTTTCCCC ATTCCCAACG TAGCCAATGC CACAGCCCGT 540

GTGGCCGACA AGGTTGTTTG GCAGACGTCC CCGAAGACGT TCAGGAAAAA CTTGCATCCG 600

CAGCATAAGA TGTTGAAGGA TACAGTTCTT GATATTATCA AGCCGGGGCA CGGAGAGTAT 660

GTGGGTTGGG GAGAGATGGG AGGCATCGAG TTTATGAAGG AGCCAACATT CATGAATTAT 720

TTCAACTTTG ACAATATGCA ATATCAGCAG GTCTATGCAC AAGGCGCTCT TGATAGTCGT 780

GAGCCGTTGT ATCACTCTGA TCCCTTCTAT CTCGACGTGA ACTCCAACCC AGAGCACAAG 840

AACATTACGG CAACCTTTAT CGATAACTAC TCTCAGATTG CCATCGACTT TGGGAAGACC 900

AACTCAGGCT ACATCAAGCT GGGTACCAGG TATGGCGGTA TCGATTGTTA CGGTATCAGC 960

GCGGATACGG TCCCGGAGAT TGTGCGACTT TATACTGGAC TTGTTGGGCG TTCGAAGTTG 1020

AAGCCCAGGT ATATTCTCGG AGCCCACCAA GCTTGTTATG GATACCAGCA GGAAAGTGAC 1080

TTGCATGCTG TTGTTCAGCA GTACCGTGAC ACCAAGTTTC CGCTTGATGG GTTGCATGTC 1140

GATGTCGACT TTCAGGACAA TTTCAGAACG TTTACCACTA ACCCGATTAC GTTCCCTAAT 1200

CCCAAAGAAA TGTTTACCAA TCTAAGGAAC AATGGAATCA AGTGTTCCAC CAACATCACC 1260

CCTGTTATCA GTATCAGAGA TCGCCCGAAT GGGTACAGTA CCCTCAATGA GGGATATGAT 1320

AAAAAGTACT TCATCATGGA TGACAGATAT ACCGAGGGGA CAAGTGGGGA CCCGCAAAAT 1380

GTTCGATACT CTTTTTACGG CGGTGGGAAC CCGGTTGAGG TTAACCCTAA TGATGTTTGG 1440

GCTCGGCCAG ACTTTGGAGA CAATTATGAC TTCCCTACGA ACTTCAACTG CAAAGACTAC 1500

CCCTATCATG GTGGTGTGAG TTACGGATAT GGGAATGGCA CTCCAGGTTA CTACCCTGAC 1560

CTTAACAGAG AGGAGGTTCG TATCTGGTGG GGATTGCAGT ACGAGTATCT CTTCAATATG 1620

GGACTAGAGT TTGTATGGCA AGATATGACA ACCCCAGCGA TCCATTCATC ATATGGAGAC 1680

ATGAAAGGGT TGCCCACCCG TCTGCTCGTC ACCGCCGACT CAGTTACCAA TGCCTCTGAG 1740

AAAAAGCTCG CAATTGAAAG TTGGGCTCTT TACTCCTACA ACCTCCATAA AGCAACCTTC 1800

CACGGTCTTG GTCGTCTTGA GTCTCGTAAG AACAAACGTA ACTTCATCCT CGGACGTGGT 1860

AGTTACGCCG GTGCCTATCG TTTTGCTGGT CTCTGGACTG GAGATAACGC AAGTACGTGG 1920

GAATTCTGGA AGATTTCGGT CTCCCAAGTT CTTTCTCTAG GTCTCAATGG TGTGTGTATA 1980

GCGGGGTCTG ATACGGGTGG TTTTGAGCCC GCACGTACTG AGATTGGGGA GGAGAAATAT 2040

TGCAGTCCGG AGCTACTCAT CAGGTGGTAT ACTGGATCAT TCCTTTTGCC ATGGCTTAGA 2100

AACCACTACG TCAAGAAGGA CAGGAAATGG TTCCAGGAAC CATACGCGTA CCCCAAGCAT 2160

CTTGAAACCC ATCCAGAGCT CGCAGATCAA GCATGGCTTT ACAAATCTGT TCTAGAAATT 2220

TGCAGATACT GGGTAGAGCT AAGATATTCC CTCATCCAGC TCCTTTACGA CTGCATGTTC 2280

CAAAACGTGG TCGATGGTAT GCCACTTGCC AGATCTATGC TCTTGACCGA TACTGAGGAT 2340

ACGACCTTCT TCAATGAGAG CCAAAAGTTC CTCGATAACC AATATATGGC TGGTGACGAC 2400

ATCCTTGTAG CACCCATCCT CCACAGCCGT AACGAGGTTC CGGGAGAGAA CAGAGATGTC 2460

TATCTCCCTC TATTCCACAC CTGGTACCCC TCAAACTTGA GACCGTGGGA CGATCAGGGA 2520

GTCGCTTTAG GGAATCCTGT CGAAGGTGGC AGCGTTATCA ACTACACTGC CAGGATTGTT 2580

GCCCCAGAGG ATTATAATCT CTTCCACAAC GTGGTGCCGG TCTACATCAG AGAGGGTGCC 2640

ATCATTCCGC AAATTCAGGT ACGCCAGTGG ATTGGCGAAG GAGGGCCTAA TCCCATCAAG 2700

TTCAATATCT ACCCTGGAAA GGACAAGGAG TATGTGACGT ACCTTGATGA TGGTGTTAGC 2760

CGCGATAGTG CACCAGATGA CCTCCCGCAG TACCGCGAGG CCTATGAGCA AGCGAAGGTC 2820

GAAGGCAAAG ACGTCCAGAA GCAACTTGCG GTCATTCAAG GGAATAAGAC TAATGACTTC 2880

TCCGCCTCCG GGATTGATAA GGAGGCAAAG GGTTATCACC GCAAAGTTTC TATCAAACAG 2940

GAGTCAAAAG ACAAGACCCG TACTGTCACC ATTGAGCCAA AACACAACGG ATACGACCCC 3000

TCTAAGGAAG TTGGTAATTA TTATACCATC ATTCTTTGGT ACGCACCGGG CTTTGACGGC 3060

AGCATCGTCG ATGTGAGCCA GGCGACCGTG AACATCGAGG GCGGGGTGGA ATGCGAAATT 3120

TTCAAGAACA CCGGCTTGCA TACGGTTGTA GTCAACGTGA AAGAGGTGAT CGGTACCACA 3180

AAGTCCGTCA AGATCACTTG CACTACCGCT TAG 3213

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr Val Leu Asp
1 5 10 15

Ile Val Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly Glu Met Gly
20 25 30

Gly Ile Gln Phe Met Lys Glu Pro Thr Phe Met Asn Tyr Phe Asn Phe
35 40 45

Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala Leu Asp Ser
50 55 60

Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr
65 70 75

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(3, "")
(D) OTHER INFORMATION: /standard_name= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
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(D) OTHER INFORMATION: /note= "N is C or T"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(3, "")
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(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(9, "")
(D) OTHER INFORMATION: /note= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(15, "")
(D) OTHER INFORMATION: /note= "N is G or A or T or C"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(18, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(21, "")
(D) OTHER INFORMATION: /note= "N is C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CANCANAANA TGCTNAANGA NAC 23

(2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(3, "")
(D) OTHER INFORMATION: /note= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(6, "")
(D) OTHER INFORMATION: /note= "N is C or T"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(9, "")
(D) OTHER INFORMATION: /note= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(15, "")
(D) OTHER INFORMATION: /note= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(18, "")
(D) OTHER INFORMATION: /note= "N is G or A"
(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(21, "")
(D) OTHER INFORMATION: /note= "N is C or T"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CANCANAANA TGTTNAANGA NAC 23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(3, "")

(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(6, "")
(D) OTHER INFORMATION: /note= "N is G or A or T or C"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(9, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(12, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(15, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(18, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TANAANGGNT CNCTNTGNTA 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(3, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(6, "")
(D) OTHER INFORMATION: /note= "N is G or A or T or C"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
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(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
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(B) LOCATION: replace(12, "")
(D) OTHER INFORMATION: /note= "N is G or A or T or C"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(15, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(18, "")
(D) OTHER INFORMATION: /note= "N is G or A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TANAANGGNT CNGANTGNTA 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAACTGCAGC TGGCGCGCCA TGGCAGGATT TTCTGAT 37

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 1

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
3 OCTOBER 1994	NCIMB 40687

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Y. Merinus-v.d. Nouweland

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 3

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
3 OCTOBER 1994	NCIMB 40688

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Authorized officer: Y. Marinus-v.d. Nouwland	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 5

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
3 OCTOBER 1994	NCIMB 40689

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Y. Marinus-v.d. Nouweland

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CLAIMS

1. A method of preparing the enzyme α-1,4-glucan lyase comprising isolating the enzyme from a culture of a fungus wherein the culture is substantially free of any other organism.

2. A method according to claim 1 wherein the enzyme is isolated and/or further purified using a gel that is not degraded by the enzyme.

3. A method according to claim 2 wherein the gel is based on dextrin or derivatives thereof, preferably a cyclodextrin, more preferably beta-cyclodextrin.

4. A method according to any one of claims 1 to 3 wherein the fungus is *Morchella costata* or *Morchella vulgaris*.

5. A GL enzyme prepared by the method according to any one of claims 1 to 4.

6. An enzyme comprising the amino acid sequence SEQ. ID. No. 1 or SEQ. I.D. No. 2, or any variant thereof.

7. A nucleotide sequence capable of coding for the enzyme α-1,4-glucan lyase.

8. A nucleotide sequence according to claim 7 wherein the sequence is a DNA sequence.

9. A nucleotide sequence according to claim 8 wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

10. A method of preparing the enzyme α-1,4-glucan lyase comprising expressing the nucleotide sequence of claim 9.

11. The use of beta-cyclodextrin to purify an enzyme, preferably GL.

12. A nucleotide sequence wherein the DNA sequence is made up of at least a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4.

Fig 1

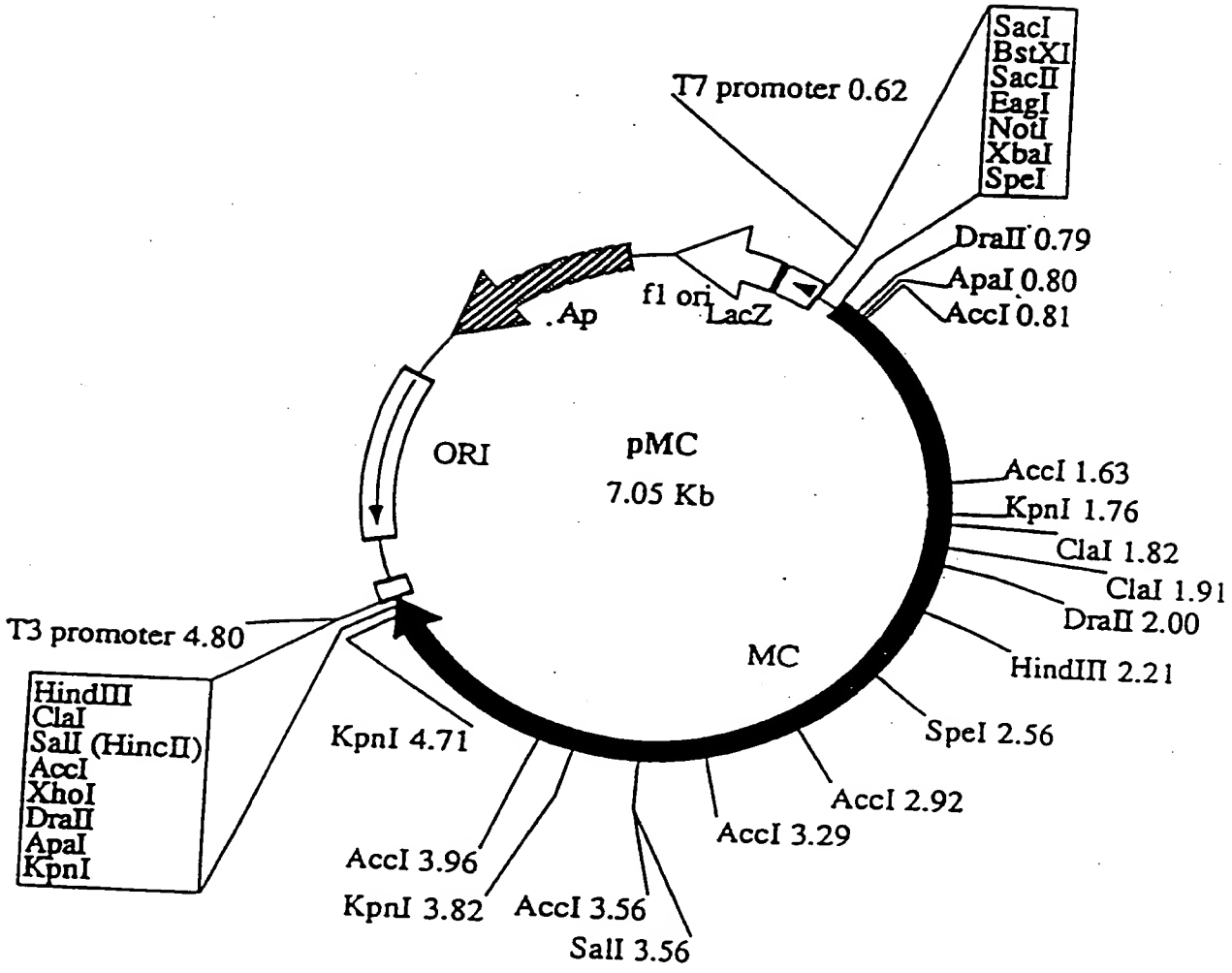

SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
T7 promoter 0.62
DraII 0.79
ApaI 0.80
AccI 0.81
f1 ori
LacZ
Ap
ORI
pMC
7.05 Kb
AccI 1.63
KpnI 1.76
ClaI 1.82
ClaI 1.91
DraII 2.00
HindIII 2.21
SpeI 2.56
AccI 2.92
AccI 3.29
AccI 3.56
SalI 3.56
KpnI 3.82
AccI 3.96
KpnI 4.71
MC
T3 promoter 4.80
HindIII
ClaI
SalI (HincII)
AccI
XhoI
DraII
ApaI
KpnI

Fig 2

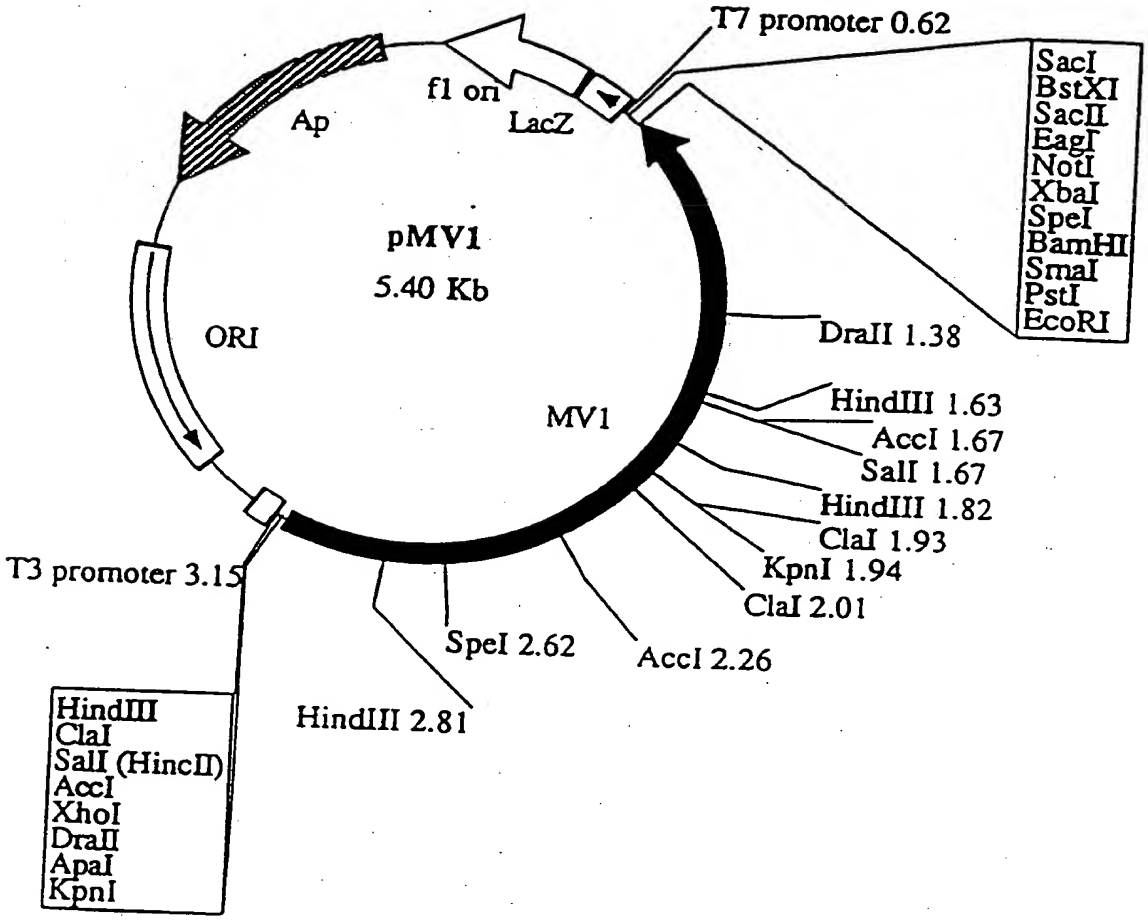
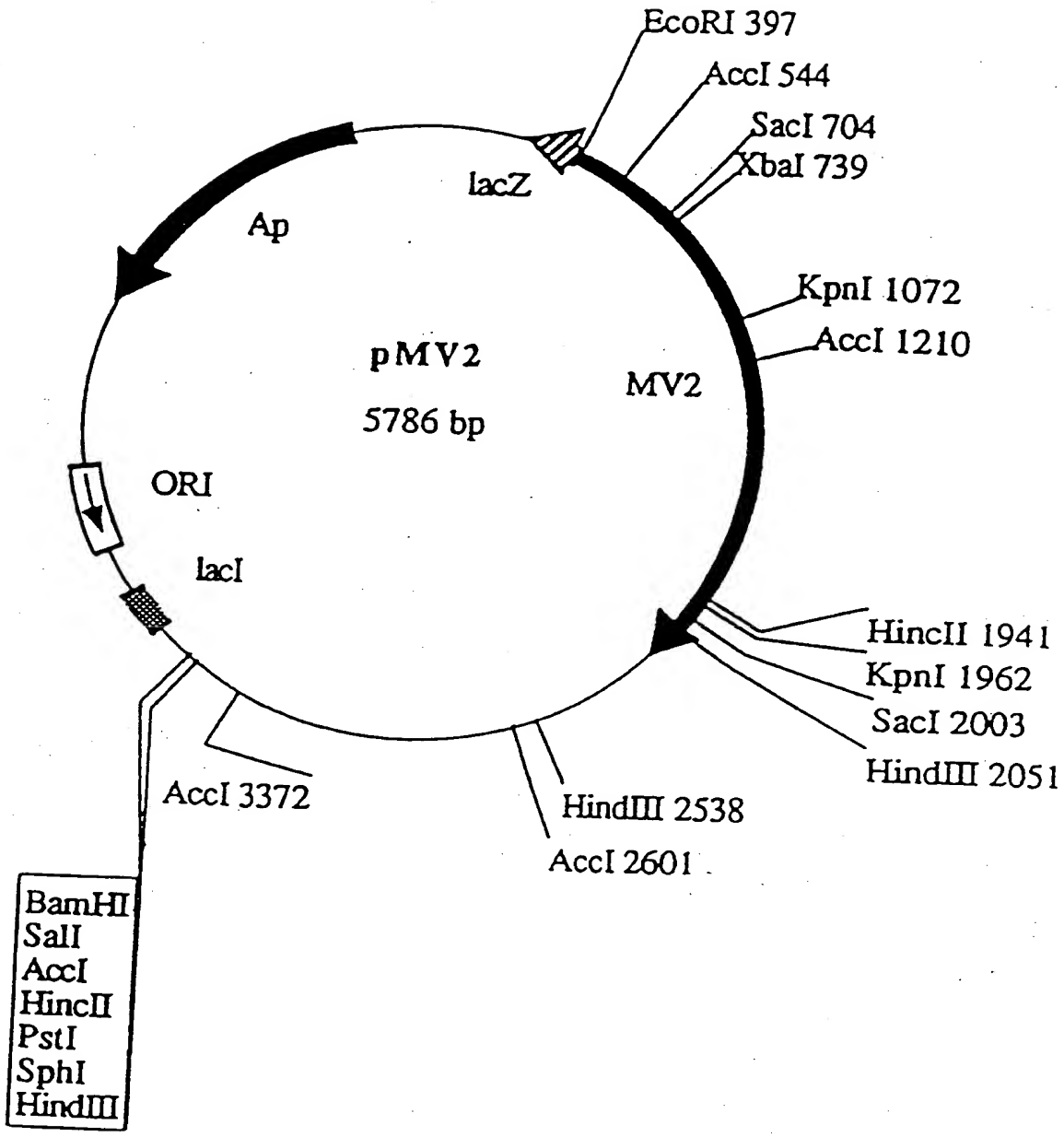

T7 promoter 0.62
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
f1 ori
Ap
LacZ
pMV1
5.40 Kb
ORI
MV1
DraII 1.38
HindIII 1.63
AccI 1.67
SalI 1.67
HindIII 1.82
ClaI 1.93
KpnI 1.94
ClaI 2.01
AccI 2.26
SpeI 2.62
HindIII 2.81
T3 promoter 3.15
HindIII
ClaI
SalI (HincII)
AccI
XhoI
DraII
ApaI
KpnI

Fig 3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AccI 544
SacI 704
XbaI 739
lacZ
Ap
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5786 bp
MV2
ORI
lacI
HincII 1941
KpnI 1962
SacI 2003
HindIII 2051
AccI 3372
HindIII 2538
AccI 2601
BamHI
SalI
AccI
HincII
PstI
SphI
HindIII

FIGURE 4

```
                  10         20         30         40         50         60
                   |          |          |          |          |          |
    1 AGACAGGTGC GTTTTTGTTT ATTCTATTCT GTGCGGCAGA TATGCACTCA CAAGAAACAA
   61 ATTGTACAAA TATTTCTAAT TACAGTTGTA GGTGCAGTTG AAAATCCGGT CGCACAAAGA
  121 TCATTGATGC ACAAAGATGA TAACGCCTGA TTAGTACTCA AGGTTTAATT GGGTATGTGT
  181 GCGACCTCTC TTTGGCTAGC ATTACCTGAT TGGTTACAAC TGCAAATACT GCGGCAGCAA
  241 TGAGGAATGA AGTCAGCATC GATAGCTCGG CCTCATAAAA ATTGATTTCA ATTTTATATT
  301 CCCAGTTTTA ATCTCGAATC CTATATAATG GCCATCGTTC CCTCCTCGCC TCTTCATTCT
  361 CCTCCATCAC TCCAGCTCAG TCATCCCTCA ACTTGGCCTC CTCTGATATC TTCCGAACAA
  421 AACATCTTGT CCAATCTTTT TTTGAGCTAG ATCTCATTAT ACCTCCGTCA TGGCAGGATT
  481 TTCTGATCCT CTCAACTTTT GCAAAGCAGA AGACTACTAC AGTGTTGCGC TAGACTGGAA
  541 GGGCCCTCAA AAAATCATTG GAGTAGACAC TACTCCTCCA AAGAGCACCA AGTTCCCCAA
  601 AAACTGGCAT GGAGTGAACT TGAGATTCGA TGATGGGACT TTAGGTGTGG TTCAGTTCAT
  661 TAGGCCGTGC GTTTGGAGGG TTAGATACGA CCCTGGTTTC AAGACCTCTG ACGAGTATGG
  721 TGATGAGAAT ACGTGAGTTA CCCCATATGT CATTATTGGT AGCGAAAAAC ATATGCTAAT
  781 CAACTAACGA GGCATATAGG AGGACAATTG TGCAAGATTA TATGAGTACT CTGAGTAATA
  841 AATTGGATAC TTATAGAGGT CTTACGTGGG AAACCAAGTG TGAGGATTCG GGAGATTTCT
  901 TTACCTTCTC AGTAAGTGCC AGTACTGCTA TAGCTCCGCT ATATATATAA CACCACTAAC
  961 TAACTGCCCT AAATAGTCCA AGGTCACCGC CGTTGAAAAA TCCGAGCGGA CCCGCAACAA
 1021 GGTCGGCGAT GGCCTCAGAA TTCACCTATG GAAAAGCCCT TTCCGCATCC AAGTAGTGCG
 1081 CACCTTGACC CCTTTGAAGG ATCCTTACCC CATTCCAAAT GTAGCCGCAG CCGAAGCCCG
 1141 TGTGTCCGAC AAGGTCGTTT GGCAAACGTC TCCCAAGACA TTCAGAAAGA ACCTGCATCC
 1201 GCAACACAAG ATGCTAAAGG ATACAGTTCT TGACATTGTC AAACCTGGAC ATGGCGAGTA
 1261 TGTGGGGTGG GGAGAGATGG GAGGTATCCA GTTTATGAAG GAGCCAACAT TCATGAACTA
 1321 TTTTAGTAAG CCCCGAAGAG GTTCCTTATA AATTCTTGGT GGTCATTTTT ACTAACCCAG
 1381 TGTAGACTTC GACAATATGC AATACCAGCA AGTCTATGCC CAAGGTGCTC TCGATTCTCG
 1441 CGAGCCACTG TAAGTACCGT CCTGTGGCAC GACTTAACCC AATAACTAAT CTTTCAACAA
 1501 GGTACCACTC GGATCCCTTC TATCTTGATG TGAACTCCAA CCCGGAGCAC AAGAATATCA
 1561 CGGCAACCTT TATCGATAAC TACTCTCAAA TTGCCATCGA CTTTGGAAAG ACCAACTCAG
```

FIGURE 4 CONTINUED

1621 GCTACATCAA GCTGGGAACC AGGTATGGTG GTATCGATTG TTACGGTATC AGTGCGGATA

1681 CGGTCCCGGA AATTGTACGA CTTTATACAG GTCTTGTTGG ACGTTCAAAG TTGAAGCCCA

1741 GATATATTCT CGGGGCCCAT CAAGCCTGTA AGTCCTTCCC CTCATGAGTG ATTTATCAGA

1801 CTTGCATAAT AAACTAACCT CGTTTTCAAA GGTTATGGAT ACCAACAGGA AAGTGACTTG

1861 TATTCTGTGG TCCAGCAGTA CCGTGACTGT AAATTTCCAC TTGACGGGAT TCACGTCGAT

1921 GTCGATGTTC AGGTAAATGG CCATGGTATC ATTGAAGCTT TGAGAAATGT TCTAACTGTG

1981 TTTATAACAT TCCTAGGACG GCTTCAGAAC TTTCACCACC AACCCACACA CTTTCCCTAA

2041 CCCCAAAGAG ATGTTTACTA ACTTGAGGAA TAATGGAATC AAGTGCTCCA CCAATATCAC

2101 TCCTGTTATC AGCATTAACA ACAGAGAGGG TGGATACAGT ACCCTCCTTG AGGGAGTTGA

2161 CAAAAAATAC TTTATCATGG ACGACAGATA TACCGAGGGA ACAAGTGGGA ATGCGAAGGA

2221 TGTTCGGTAC ATGTACTACG GTGGTGGTAA TAAGGTTGAG GTCGATCCTA ATGATGTTAA

2281 TGGTCGGCCA GACTTTAAAG ACAACTAGTA AGTTGTTTAT TTGACTACGA TAGGTAACCC

2341 GTAAGCGGCA TTAACATATT TGTAGTGACT TCCCCGCGAA CTTCAACAGC AAACAATACC

2401 CCTATCATGG TGGTGTGAGC TACGGTTATG GGAACGGTAG TGTAAGTGAC GATATCTCAC

2461 CAACATAATG AAATTTATAA GGACTAACTA GACACAAAAA TTTGTAGGCA GGTTTTTACC

2521 CGGACCTCAA CAGAAAGGAG GTTCGTATCT GGTGGGGAAT GCAGTACAAG TATCTCTTCG

2581 ATATGGGACT GGAATTTGTG TGGCAAGACA TGACTACCCC AGCAATCCAC ACATCATATG

2641 GAGACATGAA AGGGTTGCCC ACCCGTCTAC TCGTCACCTC AGACTCCGTC ACCAATGCCT

2701 CTGAGAAAAA GCTCGCAATT GAAACTTGGG CTCTCTACTC CTACAATCTC CACAAAGCAA

2761 CTTGGCATGG TCTTAGTCGT CTCGAATCTC GTAAGAACAA ACGAAACTTC ATCCTCGGGC

2821 GTGGAAGTTA TGCCGGAGCC TATCGTTTTG CTGGTCTCTG GACTGGGGAT AATGCAAGTA

2881 ACTGGGAATT CTGGAAGATA TCGGTCTCTC AAGTTCTTTC TCTGGGCCTC AATGGTGTGT

2941 GCATCGCGGG GTCTGATACG GGTGGTTTTG AACCCTACCG TGATGCAAAT GGGGTCGAGG

3001 AGAAATACTG TAGCCCAGAG CTACTCATCA GGTGGTATAC TGGTTCATTC CTCTTGCCGT

3061 GGCTCAGGAA CCATTATGTC AAAAAGGACA GGAAATGGTT CCAGGTAATC TATCCTTTCT

3121 TATCTTTGAA GCATTGAAGA TACTAAGATA TAATCTAGGA ACCATACTCG TACCCCAAGC

3181 ATCTTGAAAC CCATCCAGAA CTCGCAGACC AAGCATGGCT CTATAAATCC GTTTTGGAGA
3241 TCTGTAGGTA CTATGTGGAG CTTAGATACT CCCTCATCCA ACTACTTTAC GACTGCATGT

FIGURE 4 CONTINUED

3301 TTCAAAACGT AGTCGACGGT ATGCCAATCA CCAGATCTAT GGTATGTATT CTACCCTAGG

3361 CTTCCAGAGC AACATATGCT AACCAATTGA ACCTGGGTTT CTAGCTCTTG ACCGATACTG

3421 AGGATACCAC CTTCTTCAAC GAGAGCCAAA AGTTCCTCGA CAACCAATAT ATGGCTGGTG

3481 ACGACATTCT TGTTGCACCC ATCCTCCACA GTCGCAAAGA AATTCCAGGC GAAAACAGAG

3541 ATGTCTATCT CCCTCTTTAC CACACCTGGT ACCCCTCAAA TTTGAGACCA TGGGACGATC

3601 AAGGAGTCGC TTTGGGGAAT CCTGTCGAAG GTGGTAGTGT CATCAATTAT ACTGCTAGGA

3661 TTGTTGCACC CGAGGATTAT AATCTCTTCC ACAGCGTGGT ACCAGTCTAC GTTAGAGAGG

3721 GTAAGCAGTA AAATAATCTC TTCCCAGTTT CAAATACATT TAGCTAGTAG CTAACGCTAT

3781 GAACCTACAG GTGCCATCAT CCCGCAAATC GAAGTACGCC AATGGACTGG CCAGGGGGGA

3841 GCCAACCGCA TCAAGTTCAA CATCTACCCT GGAAAGGATA AGGTAAAATT CAATGATCAC

3901 CCTGCATCTA TTCCATCGCT GGTTTTCTTT ACCCTTACTG ACTTCATTCC TCAAAATACA

3961 GGAGTACTGT ACCTATCTTG ATGATGGTGT TAGCCGTGAT AGTGCGCCGG AAGACCTCCC

4021 ACAGTACAAA GAGACCCACG AACAGTCGAA GGTTGAAGGC GCGGAAATCG CAAAGCAGAT

4081 TGGAAAGAAG ACGGGTTACA ACATCTCAGG AACCGACCCA GAAGCAAAGG GTTATCACCG

4141 CAAAGTTGCT GTCACACAAG TAATACCGCC CTTGACTTGT ATCACTTCCT GACATCATGC

4201 TAATATTTCT CTGTTTACCT CAAAGACGTC AAAAGACAAG ACGCGTACTG TCACTATTGA

4261 GCCAAAACAC AATGGATACG ACCCTTCCAA AGAGGTGGGT GATTATTATA CCATCATTCT

4321 TTGGTACGCA CCAGGTTTCG ATGGCAGCAT CGTCGATGTG AGCAAGACGA CTGTGAATGT

4381 TGAGGGTGGG GTGGAGCACC AAGTTTATAA GAACTCCGAT TTACATACGG TTGTTATCGA

4441 CGTGAAGGAG GTGATCGGTA CCACAAAGAG CGTCAAGATC ACATGTACTG CCGCTTAAGG

4501 TCTTTTCTTG GGGGCGGGAG GCGAGACCTT CGAAATGTAT ACGGGAGTGG TAACTCCGGG

4561 AAAATGGTGA TATGGGGGAT CAAGTTGGAG GGGAATCTGT TTATTTCTTT ATTTCTTTAT

4621 TTACTGGATT GGAAAATAGG GAGCACAGTT CTGACTGGAT TGGTTTGATT GTTGGCCTCT

4681 ACGGGTTCTC TTTACTTTGT CTGGAAATCC AATTTATTGT TATGCG

FIGURE 5

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                  10        20        30        40        50        60
                   |         |         |         |         |         |
   1 ATGCAGGCAA CGACAGGCGT TTTTTGTTTT ATCCGCAGAG GTGCAGCAGC AGGAAACAAA
  61 CCATACAAAC ATTCCTTGAC GCGGTTTTAG GTGCAGTTAA GGCCCGGGCG CACCAAGAAC
 121 ATTGATGTAC TTGGTCTAAA AAAGATCATA ATACCCGATT AGTGTTCATG GTTTGATTGG
 181 GTCTAAGTAC AAGTTTTACA GAGTTCAGCT TAGTTCATTG TTCGAAACTA CCAATATCAC
 241 ACCTATGCCT GCTGGCATTG ATAGCTCGGC TTGTGAAAGC TGATTACAAT CTTACATTTC
 301 TGATTTAATA TCGGACTGAT CTATATATAA GGGTCATCAT TTCCTCTCCG CCTTTTGGTT
 361 CTCTTTCATC ACCCCAGCCC AATCATCACC GTTGGCCTTT ACTTCTCTCT TCCGTTGATA
 421 TTTTCTCGAC AAAACATCTT GTCCACTGTT AGGCTAGCTC CCAGAATTAT CCCTCCAACA
 481 TGGCAGGATT ATCCGACCCT CTCAATTTCT GCAAAGCAGA GGACTACTAC GCTGCTGCCA
 541 AAGGCTGGAG TGGCCCTCAG AAGATCATTC GCTATGACCA GACCCCTCCT CAGGGTACAA
 601 AAGATCCGAA AAGCTGGCAT GCGGTAAACC TTCCTTTCGA TGACGGGACT ATGTGTGTAG
 661 TGCAATTCGT CAGACCCTGT GTTTGGAGGG TTAGATATGA CCCCAGTGTC AAGACTTCTG
 721 ATGAGTACGG CGATGAGAAT ACGTGGGTCG CCCAGTCAAT TAACTATGCC GCTAGTGATT
 781 ATGGAAAGCT TCTGCTAACC GATCAATGAG GCATGTAGGA GGACTATTGT ACAAGACTAC
 841 ATGACTACTC TGGTTGGAAA CTTGGACATT TTCAGAGGTC TTACGTGGGT TTCTACGTTG
 901 GAGGATTCGG GCGAGTACTA CACCTTCAAG GCAAGCCTCA GTGTTATATC TCGAATATAT
 961 TATATATCAC AACAAACTAA CTAGTCATAC AGTCCGAAGT CACTGCCGTG GACGAAACCG
1021 AACGGACTCG AAACAAGGTC GGCGACGGCC TCAAGATTTA CCTATGGAAA AATCCCTTTC
1081 GCATCCAGGT AGTGCGTCTC TTGACCCCCC TGGTGGACCC TTTCCCCATT CCCAACGTAG
1141 CCAATGCCAC AGCCCGTGTG GCCGACAAGG TTGTTTGGCA GACGTCCCCG AAGACGTTCA
1201 GGAAAAACTT GCATCCGCAG CATAAGATGT TGAAGGATAC AGTTCTTGAT ATTATCAAGC
1261 CGGGGCACGG AGAGTATGTG GGTTGGGGAG AGATGGGAGG CATCGAGTTT ATGAAGGAGC
1321 CAACATTCAT GAATTATTTC AGTAAGCTCT TGAAAGATTT CCTATCTCTT GACGGTCGTT
1381 TTTGCTAAGG AAACTGTAGA CTTTGACAAT ATGCAATATC AGCAGGTCTA TGCACAAGGC
1441 GCTCTTGATA GTCGTGAGCC GTTGTAAGTA ACGTCCTGTG ACATGTCATG ATTACAGTAA
1501 CTGATCGTTC AATAAGGTAT CACTCTGATC CCTTCTATCT CGACGTGAAC TCCAACCCAG
1561 AGCACAAGAA CATTACGGCA ACCTTTATCG ATAACTACTC TCAGATTGCC ATCGACTTTG
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FIGURE 5 CONTINUED

1621 GGAAGACCAA CTCAGGCTAC ATCAAGCTGG GTACCAGGTA TGGCGGTATC GATTGTTACG

1681 GTATCAGCGC GGATACGGTC CCGGAGATTG TGCGACTTTA TACTGGACTT GTTGGGCGTT

1741 CGAAGTTGAA GCCCAGGTAT ATTCTCGGAG CCCACCAAGC TTGTAAGCCC GCCCCCTTTA

1801 CGATGCATTT ATTAGGGGTC CACAGACTAA ACTTGTTCCA AAGGTTATGG ATACCAGCAG

1861 GAAAGTGACT TGCATGCTGT TGTTCAGCAG TACCGTGACA CCAAGTTTCC GCTTGATGGG

1921 TTGCATGTCG ATGTCGACTT TCAGGTAAAT GGCCCAGGTA TCGTTGAAGC TTTGGAGAAT

1981 GCTAATTGTG CTCGTAAAAC TTTAAGGACA ATTTCAGAAC GTTTACCACT AACCCGATTA

2041 CGTTCCCTAA TCCCAAAGAA ATGTTTACCA ATCTAAGGAA CAATGGAATC AAGTGTTCCA

2101 CCAACATCAC CCCTGTTATC AGTATCAGAG ATCGCCCGAA TGGGTACAGT ACCCTCAATG

2161 AGGGATATGA TAAAAAGTAC TTCATCATGG ATGACAGATA TACCGAGGGG ACAAGTGGGG

2221 ACCCGCAAAA TGTTCGATAC TCTTTTTACG GCGGTGGGAA CCCGGTTGAG GTTAACCCTA

2281 ATGATGTTTG GGCTCGGCCA GACTTTGGAG ACAATTAGTA AGTTACTCAA TAGGCTACTT

2341 GAGATATTCT GTAGGTGGCA TTAACACGAC TATAGTGACT TCCCTACGAA CTTCAACTGC

2401 AAAGACTACC CCTATCATGG TGGTGTGAGT TACGGATATG GGAATGGCAC TGTAAGTGAT

2461 AATAAGTCAT AAATACAACG TAATTCATGG AGACTAATCA GTGGTAAATG AATTTTAGCC

2521 AGGTTACTAC CCTGACCTTA ACAGAGAGGA GGTTCGTATC TGGTGGGGAT TGCAGTACGA

2581 GTATCTCTTC AATATGGGAC TAGAGTTTGT ATGGCAAGAT ATGACAACCC CAGCGATCCA

2641 TTCATCATAT GGAGACATGA AAGGGTTGCC CACCCGTCTG CTCGTCACCG CCGACTCAGT

2701 TACCAATGCC TCTGAGAAAA AGCTCGCAAT TGAAAGTTGG GCTCTTTACT CCTACAACCT

2761 CCATAAAGCA ACCTTCCACG GTCTTGGTCG TCTTGAGTCT CGTAAGAACA AACGTAACTT

2821 CATCCTCGGA CGTGGTAGTT ACGCCGGTGC CTATCGTTTT GCTGGTCTCT GGACTGGAGA

2881 TAACGCAAGT ACGTGGGAAT TCTGGAAGAT TTCGGTCTCC CAAGTTCTTT CTCTAGGTCT

2941 CAATGGTGTG TGTATAGCGG GGTCTGATAC GGGTGGTTTT GAGCCCGCAC GTACTGAGAT

3001 TGGGGAGGAG AAATATTGCA GTCCGGAGCT ACTCATCAGG TGGTATACTG GATCATTCCT

3061 TTTGCCATGG CTTAGAAACC ACTACGTCAA GAAGGACAGG AAATGGTTCC AGGTAATATA

3121 CTCTTTCTGG TCTCTGAGTA TCGAAGACGC TAAGACAATA TAGGAACCAT ACGCGTACCC

3181 CAAGCATCTT GAAACCCATC CAGAGCTCGC AGATCAAGCA TGGCTTTACA AATCTGTTCT

3241 AGAAATTTGC AGATACTGGG TAGAGCTAAG ATATTCCCTC ATCCAGCTCC TTTACGACTG

FIGURE 5 CONTINUED

3301 CATGTTCCAA AACGTGGTCG ATGGTATGCC ACTTGCCAGA TCTATGGTAT GCATTTTATC

3361 CGTCTCCTTT CACGATAATG CACCAGTCTA ACCGAATTTT CTTTTAGCTC TTGACCGATA

3421 CTGAGGATAC GACCTTCTTC AATGAGAGCC AAAAGTTCCT CGATAACCAA TATATGGCTG

3481 GTGACGACAT CCTTGTAGCA CCCATCCTCC ACAGCCGTAA CGAGGTTCCG GGAGAGAACA

3541 GAGATGTCTA TCTCCCTCTA TTCCACACCT GGTACCCCTC AAACTTGAGA CCGTGGGACG

3601 ATCAGGGAGT CGCTTTAGGG AATCCTGTCG AAGGTGGCAG CGTTATCAAC TACACTGCCA

3661 GGATTGTTGC CCCAGAGGAT TATAATCTCT TCCACAACGT GGTGCCGGTC TACATCAGAG

3721 AGGGTAAGCG ATGGAATAAT TTCTTGCAAG TTCCAGATAC AAGTGGTTAC TGACACCTTA

3781 AACCAGGTGC CATCATTCCG CAAATTCAGG TACGCCAGTG GATTGGCGAA GGAGGGCCTA

3841 ATCCCATCAA GTTCAATATC TACCCTGGAA AGGACAAGGT ATATTCTCCA TGACTATCGC

3901 GCATTTATTC TTTCTCTACT CGCACTAACT TCATCTGAAT ATAGGAGTAT GTGACGTACC

3961 TTGATGATGG TGTTAGCCGC GATAGTGCAC CAGATGACCT CCCGCAGTAC CGCGAGGCCT

4021 ATGAGCAAGC GAAGGTCGAA GGCAAAGACG TCCAGAAGCA ACTTGCGGTC ATTCAAGGGA

4081 ATAAGACTAA TGACTTCTCC GCCTCCGGGA TTGATAAGGA GGCAAAGGGT TATCACCGCA

4141 AAGTTTCTAT CAAACAGGTA CATGATTTCA TCTTCCTTTT TTCGCAGTCA CTATTATATC

4201 ATCCTAACAT TGCTTCTCTT ATTTAAAAGG AGTCAAAAGA CAAGACCCGT ACTGTCACCA

4261 TTGAGCCAAA ACACAACGGA TACGACCCCT CTAAGGAAGT TGGTAATTAT TATACCATCA

4321 TTCTTTGGTA CGCACCGGGC TTTGACGGCA GCATCGTCGA TGTGAGCCAG GCGACCGTGA

4381 ACATCGAGGG CGGGGTGGAA TGCGAAATTT TCAAGAACAC CGGCTTGCAT ACGGTTGTAG

4441 TCAACGTGAA AGAGGTGATC GGTACCACAA AGTCCGTCAA GATCACTTGC ACTACCGCTT

4501 AGAGCTCTTT TATGAGGGGT ATATGGGAGT GGCAGCTCAG AAATTTGGGA AGCTTCTGGG

4561 TATTCCTTTT GTTTATTTAC TTATTTATTG AATCGACCAA TACGGGTGGG ATTCTCTCTG

4621 GTTTTTGTGA GGCTATGTTT TACTTGGTCT GAAAATCAAA TTCGTTCTCA

FIGURE 6

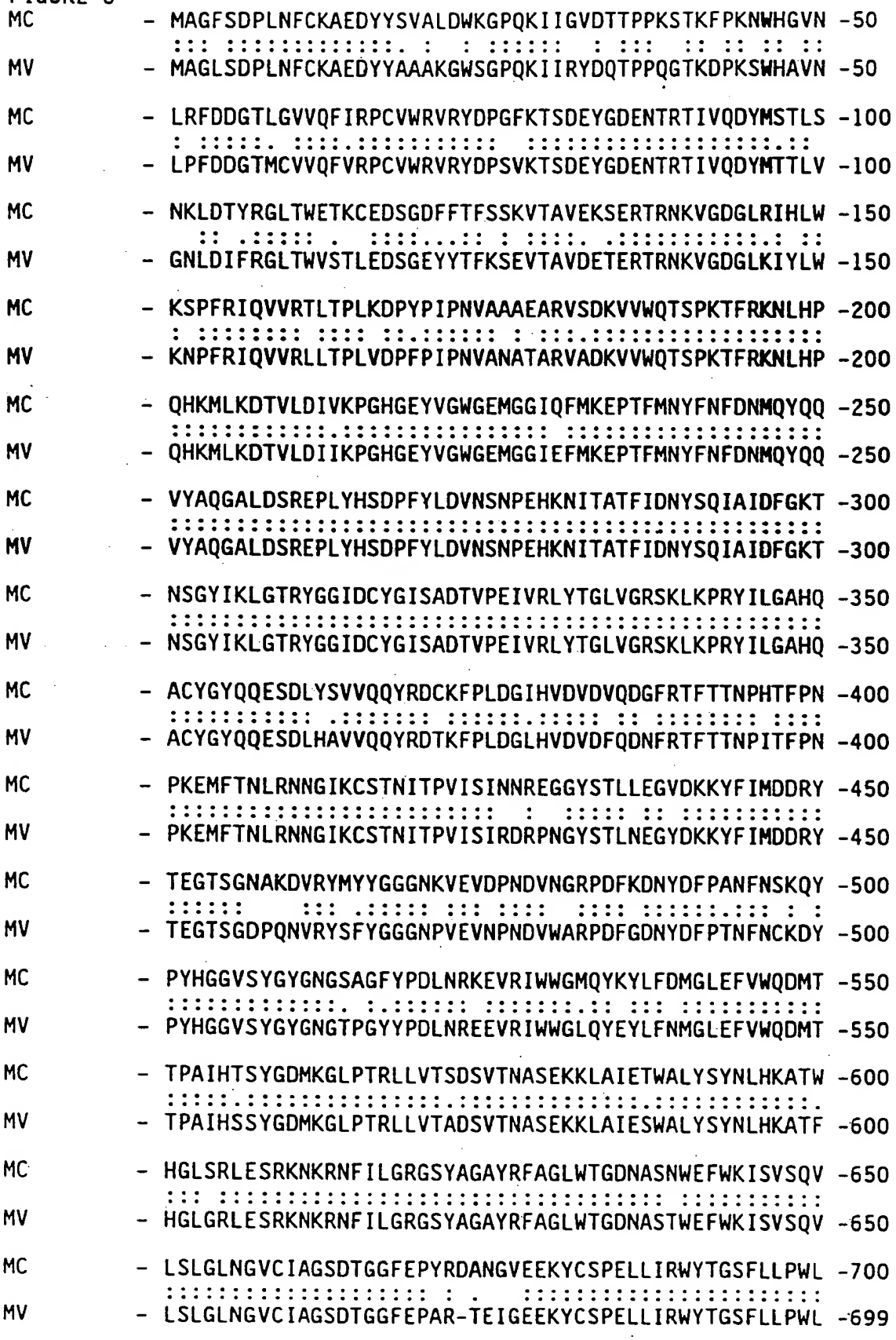
MC - MAGFSDPLNFCKAEDYYSVALDWKGPQKIIGVDTTPPKSTKFPKNWHGVN -50
MV - MAGLSDPLNFCKAEDYYAAAKGWSGPQKIIRYDQTPPQGTKDPKSWHAVN -50
MC - LRFDDGTLGVVQFIRPCVWRVRYDPGFKTSDEYGDENTRTIVQDYMSTLS -100
MV - LPFDDGTMCVVQFVRPCVWRVRYDPSVKTSDEYGDENTRTIVQDYMTTLV -100
MC - NKLDTYRGLTWETKCEDSGDFFTFSSKVTAVEKSERTRNKVGDGLRIHLW -150
MV - GNLDIFRGLTWVSTLEDSGEYYTFKSEVTAVDETERTRNKVGDGLKIYLW -150
MC - KSPFRIQVVRTLTPLKDPYPIPNVAAAEARVSDKVVWQTSPKTFRKNLHP -200
MV - KNPFRIQVVRLLTPLVDPFPIPNVANATARVADKVVWQTSPKTFRKNLHP -200
MC - QHKMLKDTVLDIVKPGHGEYVGWGEMGGIQFMKEPTFMNYFNFDNMQYQQ -250
MV - QHKMLKDTVLDIIKPGHGEYVGWGEMGGIEFMKEPTFMNYFNFDNMQYQQ -250
MC - VYAQGALDSREPLYHSDPFYLDVNSNPEHKNITATFIDNYSQIAIDFGKT -300
MV - VYAQGALDSREPLYHSDPFYLDVNSNPEHKNITATFIDNYSQIAIDFGKT -300
MC - NSGYIKLGTRYGGIDCYGISADTVPEIVRLYTGLVGRSKLKPRYILGAHQ -350
MV - NSGYIKLGTRYGGIDCYGISADTVPEIVRLYTGLVGRSKLKPRYILGAHQ -350
MC - ACYGYQQESDLYSVVQQYRDCKFPLDGIHVDVDVQDGFRTFTTNPHTFPN -400
MV - ACYGYQQESDLHAVVQQYRDTKFPLDGLHVDVDFQDNFRTFTTNPITFPN -400
MC - PKEMFTNLRNNGIKCSTNITPVISINNREGGYSTLLEGVDKKYFIMDDRY -450
MV - PKEMFTNLRNNGIKCSTNITPVISIRDRPNGYSTLNEGYDKKYFIMDDRY -450
MC - TEGTSGNAKDVRYMYYGGGNKVEVDPNDVNGRPDFKDNYDFPANFNSKQY -500
MV - TEGTSGDPQNVRYSFYGGGNPVEVNPNDVWARPDFGDNYDFPTNFNCKDY -500
MC - PYHGGVSYGYGNGSAGFYPDLNRKEVRIWWGMQYKYLFDMGLEFVWQDMT -550
MV - PYHGGVSYGYGNGTPGYYPDLNREEVRIWWGLQYEYLFNMGLEFVWQDMT -550
MC - TPAIHTSYGDMKGLPTRLLVTSDSVTNASEKKLAIETWALYSYNLHKATW -600
MV - TPAIHSSYGDMKGLPTRLLVTADSVTNASEKKLAIESWALYSYNLHKATF -600
MC - HGLSRLESRKNKRNFILGRGSYAGAYRFAGLWTGDNASNWEFWKISVSQV -650
MV - HGLGRLESRKNKRNFILGRGSYAGAYRFAGLWTGDNASTWEFWKISVSQV -650
MC - LSLGLNGVCIAGSDTGGFEPYRDANGVEEKYCSPELLIRWYTGSFLLPWL -700
MV - LSLGLNGVCIAGSDTGGFEPAR-TEIGEEKYCSPELLIRWYTGSFLLPWL -699

FIGURE 6 CONTINUED

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MC      - RNHYVKKDRKWFQEPYSYPKHLETHPELADQAWLYKSVLEICRYYVELRY -750
MV      - RNHYVKKDRKWFQEPYAYPKHLETHPELADQAWLYKSVLEICRYWVELRY -749

MC      - SLIQLLYDCMFQNVVDGMPITRSMLLTDTEDTTFFNESQKFLDNQYMAGD -800
MV      - SLIQLLYDCMFQNVVDGMPLARSMLLTDTEDTTFFNESQKFLDNQYMAGD -799

MC      - DILVAPILHSRKEIPGENRDVYLPLYHTWYPSNLRPWDDQGVALGNPVEG -850
MV      - DILVAPILHSRNEVPGENRDVYLPLFHTWYPSNLRPWDDQGVALGNPVEG -849

MC      - GSVINYTARIVAPEDYNLFHSVVPVYVREGAIIPQIEVRQWTGQGGANRI -900
MV      - GSVINYTARIVAPEDYNLFHNVVPVYIREGAIIPQIQVRQWIGEGGPNPI -899

MC      - KFNIYPGKDKEYCTYLDDGVSRDSAPEDLPQYKETHEQSKVEGAEIAKQI -950
MV      - KFNIYPGKDKEYVTYLDDGVSRDSAPDDLPQYREAYEQAKVEGKDVQKQL -949

MC      - G-----KKTGYNISGTDPEAKGYHRKVAVTQTSKDKTRTVTIEPKHNGYD -995
MV      - AVIQGNKTNDFSASGIDKEAKGYHRKVSIKQESKDKTRTVTIEPKHNGYD -999

MC      - PSKEVGDYYTIILWYAPGFDGSIVDVSKTTVNVEGGVEHQVYKNSDLHTV -1045
MV      - PSKEVGNYYTIILWYAPGFDGSIVDVSQATVNIEGGVECEIFKNTGLHTV -1049

MC      - VIDVKEVIGTTKSVKITCTAA -1066
MV      - VVNVKEVIGTTKSVKITCTTA -1070
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FIGURE 7

MAGFSDPLNF CKAEDYYSVA LDWKGPQKII GVDTTPPKST KFPKNWHGVN LRFDDGTLGV VQFIRPCVWR
VRYDPGFKTS DEYGDENTRT IVQDYMSTLS NKLDTYRGLT WETKCEDSGD FFTFSSKVTA VEKSERTRNK
VGDGLRIHLW KSPFRIQVVR TLTPLKDPYP IPNVAAAEAR VSDKVVWQTS PKTFRKNLHP QHKMLKDTVL
DIVKPGHGEY VGWGEMGGIQ FMKEPTFMNY FNFDNMQYQQ VYAQGALDSR EPLYHSDPFY LDVNSNPEHK
NITATFIDNY SQIAIDFGKT NSGYIKLGTR YGGIDCYGIS ADTVPEIVRL YTGLVGRSKL KPRYILGAHQ
ACYGYQQESD LYSVVQQYRD CKFPLDGIHV DVDVQDGFRT FTTNPHTFPN PKEMFTNLRN NGIKCSTNIT
PVISINNREG GYSTLLEGVD KKYFIMDDRY TEGTSGNAKD VRYMYYGGGN KVEVDPNDVN GRPDFKDNYD
FPANFNSKQY PYHGGVSYGY GNGSAGFYPD LNRKEVRIWW GMQYKYLFDM GLEFVWQDMT TPAIHTSYGD
MKGLPTRLLV TSDSVTNASE KKLAIETWAL YSYNLHKATW HGLSRLESRK NKRNFILGRG SYAGAYRFAG
LWTGDNASNW EFWKISVSQV LSLGLNGVCI AGSDTGGFEP YRDANGVEEK YCSPELLIRW YTGSFLLPWL
RNHYVKKDRK WFQEPYSYPK HLETHPELAD QAWLYKSVLE ICRYYVELRY SLIQLLYDCM FQNVVDGMPI
TRSMLLTDTE DTTFFNESQK FLDNQYMAGD DILVAPILHS RKEIPGENRD VYLPLYHTWY PSNLRPWDDQ
GVALGNPVEG GSVINYTARI VAPEDYNLFH SVVPVYVREG AIIPQIEVRQ WTGQGGANRI KFNIYPGKDK
EYCTYLDDGV SRDSAPEDLP QYKETHEQSK VEGAEIAKQI GKKTGYNISG TDPEAKGYHR KVAVTQTSKD
KTRTVTIEPK HNGYDPSKEV GDYYTIILWY APGFDGSIVD VSKTTVNVEG GVEHQVYKNS DLHTVVIDVK
EVIGTTKSVK ITCTAA

FIGURE 8

MAGLSDPLNF RKAEDYYAAA KGWSGPQKII RYDQTPPQGT KDPKSWHAVN LPFDDGTMCV VQFVRPCVWR
VRYDPSVKTS DEYGDENTRT IVQDYMTTLV GNLDIFRGLT WVSTLEDSGE YYTFKSEVTA VDETERTRNK
VGDGLKIYLW KNPFRIQVVR LLTPLVDPFP IPNVANATAR VADKVVWQTS PKTFRKNLHP QHKMLKDTVL
DIIKPGHGEY VGWGEMGGIE FMKEPTFMNY FNFDNMQYQQ VYAQGALDSR EPLYHSDPFY LDVNSNPEHK
NITATFIDNY SQIAIDFGKT NSGYIKLGTR YGGIDCYGIS ADTVPEIVRL YTGLVGRSKL KPRYILGAHQ
ACYGYQQESD LHAVVQQYRD TKFPLDGLHV DVDFQDNFRT FTTNPITFPN PKEMFTNLRN NGIKCSTNIT
PVISIRDRPN GYSTLNEGYD KKYFIMDDRY TEGTSGDPQN VRYSFYGGGN PVEVNPNDVW ARPDFGDNYD
FPTNFNCKDY PYHGGVSYGY GNGTPGYYPD LNREEVRIWW GLQYEYLFNM GLEFVWQDMT TPAIHSSYGD
MKGLPTRLLV TADSVTNASE KKLAIESWAL YSYNLHKATF HGLGRLESRK NKRNFILGRG SYAGAYRFAG
LWTGDNASTW EFWKISVSQV LSLGLNGVCI AGSDTGGFEP ARTEIGEEKY CSPELLIRWY TGSFLLPWLR
NHYVKKDRKW FQEPYAYPKH LETHPELADQ AWLYKSVLEI CRYWVELRYS LIQLLYDCMF QNVVDGMPLA
RSMLLTDTED TTFFNESQKF LDNQYMAGDD ILVAPILHSR NEVPGENRDV YLPLFHTWYP SNLRPWDDQG
VALGNPVEGG SVINYTARIV APEDYNLFHN VVPVYIREGA IIPQIQVRQW IGEGGPNPIK FNIYPGKDKE
YVTYLDDGVS RDSAPDDLPQ YREAYEQAKV EGKDVQKQLA VIQGNKTNDF SASGIDKEAK GYHRKVSIKQ
ESKDKTRTVT IEPKHNGYDP SKEVGNYYTI ILWYAPGFDG SIVDVSQATV NIEGGVECEI FKNTGLHTVV
VNVKEVIGTT KSVKITCTTA

INTERNATIONAL SEARCH REPORT

Intern· ·al Application No
PCT/EP 94/03398

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N9/88 C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR,A,2 617 502 (SOCIÉTÉ NATIONALE ELF AQUITAINE) 6 January 1989 cited in the application see page 5, line 16 - line 33 see claims; examples ---	5,6
A	BIOCHIMICA ET BIOPHYSICA ACTA, vol.1156, no.3, March 1993 pages 313 - 320 S.YU ET AL 'Alpha-1,4-glucan lyase , a new class of starch / glycogen degrading enzyme. I. efficient purification and characterization from red seaweeds' cited in the application see page 314, left column, paragraph 3 - page 315, left column see page 315, right column, paragraph 3 --- -/--	1,2,5,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 1995	29.05.95

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Intern· ·al Application No
PCT/EP 94/03398

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PCT/EP 94/03398

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